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Protective Effect of Omega-3 and the Potential of Toll-like Receptor Gene Expression in Rats with Doxorubicin-induced Cardiac Toxicity

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ABSTRACT

Despite the clinical signs of doxorubicin (DOX)-induced cardiomyopathy, the mechanisms underlying DOX-induced cardiac damage are unknown. Toll-like receptors (TLRs) allow cardiomyocytes to respond to either endogenous or external signals, both of which have the potential to alter the pathophysiological responses to dilated cardiomyopathy. Omega-3 (OMG-3) precursors are highly active metabolites with numerous therapeutic benefits in the prevention and/or treatment of a variety of diseases. Thus, this study was aimed at evaluating the preventive effect of omega-3 on rats suffering from acute DOX-induced cardiotoxicity associated with TLR gene expression. Thirty rats were divided into five equal groups; Group 1 received no treatment, Group 2 received doxorubicin (at a toxic dose of 20 mg/kg), and Groups 3-5 received doxorubicin (20 mg/kg) after receiving OMG-3 at various doses (100, 200, and 400 mg/kg/day, respectively) for 4 weeks. At the end of the experiment, blood samples were obtained from the heart. The real-time polymerase chain reaction (RT-PCR) was used to assess TLR2 and TLR4 gene expression. TLR2 had a significantly ($p<0.01$) elevated fold change in the DOX alone group (28.74±4.95), whereas TLR2 expression was significantly reduced in the OMG-3 pretreated groups (1.633±0.51, 0.733±0.13, and 0.709±0.16, respectively). TLR4 fold change in the DOX alone group was 8.57±1.22, whereas TLR4 expression was significantly reduced in the OMG-3 pretreated groups (100, 200, and 400 mg/kg) with values of 0.809±0.32, 0.852±0.50, and 0.272±0.16, respectively. The findings of this study revealed that OMG-3 decreased doxorubicin-induced cardiotoxicity and showed a significant cardioprotective effect by lowering TLR2 and TLR4 gene expression.

Keywords: Cardioprotective, Cardiotoxicity, Doxorubicin, Omega-3, TLR2, TLR4.

Introduction

Anthracyclines, which are first-line chemotherapeutic drugs that are used to effectively treat a wide range of cancers (including breast, prostate, lymphomas, and leukemia), are particularly hazardous to the cardiovascular system. Anthracycline chemotherapy drugs that cause cardio-cytotoxicity include daunorubicin, doxorubicin (DOX), epirubicin, idarubicin, mitoxantrone, and valrubicin.[1] Doxorubicin is the parent compound. Idarubicin is structurally similar to DOX, with the exception that ring D lacks the 4-methoxy group found in DOX. These substances have the potential to cure a wide range of disorders, including leukemia, lymphomas, stomach, uterine, ovarian, and bladder cancers, as well as lung cancer.[2] Doxorubicin is a powerful anthracycline anticancer drug with a broad spectrum of activity when used to treat cancer.[3] The toxicity of DOX to cardiac tissues limits its clinical relevance.[4-6] As a result, the antioxidant and naturally occurring chemical protective capabilities against DOX-induced cardiotoxicity have recently received a lot of attention.[7]

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The innate immune system is considered to be the body's primary defense against infectious pathogens that enter the body from outside (n = 8). Doxorubicin enhances cytokine production and boosts the activity of natural killer (NK) cells.[9]

Cytotoxic T-lymphocyte (CTL) responses are stimulated, and macrophage differentiation is promoted.[11] All of them are important components of both innate and adaptive immune responses, and they all cause direct cardiac damage.[12] The cardiotoxicity of DOX is often related to the activation of the body's innate immune system and the consequences that ensue. Toll-like receptors (TLRs) 2 and 4 are among the TLRs expressed in cardiomyocytes. These TLRs enable cardiomyocytes to respond to either endogenous or exogenous signals, which can change the pathophysiological responses to dilated cardiomyopathy. TLR2 and TLR4 expression and activation are both upregulated in experimental and *in vivo* (patients with hypertension or clinical heart failure) models. Therefore, suppressing TLR signaling may have a considerable therapeutic effect in the treatment of dilated cardiomyopathy (also known as CMD), particularly when DOX is administered.[13] As a result, TLRs are proposed to have a role in the pathophysiology of doxorubicin-induced heart failure. Omega-3 fatty acids (OMG-3) are among the most commonly recommended supplements, with a substantial global market behind them. In 2011, people spent approximately $25 billion on omega-3 supplements. These supplements have been used to treat a wide range of medical conditions, including gastrointestinal, rheumatic, mental, metabolic, renal, dermatologic, and pulmonary disorders. They are most commonly used for the primary and secondary prevention of cardiovascular disease.

The present study was conducted to investigate the potential protective benefits of omega-3 fatty acid pretreatment on rats suffering from acute cardiotoxicity caused by DOX in relation to pro-inflammatory TLR-2 and TLR-4 biomarkers.

Materials and Methods

Ethical approval
Ethical approval for this study was obtained from the ethics committee of the College of Pharmacy, Al-Mustansiriyah University, Baghdad, Iraq. The research was conducted from October 2021 to April 2022.

Source of animals
Thirty adult female Wistar rats weighing 200–200 g and aged 16 weeks were used in this study. The animals were obtained from the Animal Care and Research Unit, College of Pharmacy, Mustansiriyah University, Baghdad, Iraq. The animals were kept in standardized conditions that included chow, tap water, ad libitum, as well as a 12/12-hour light/dark cycle, a temperature of 22.5ºC, and 50-55% humidity.

Experimental groupings
The Wistar rats were randomly divided into five groups, each consisting of six animals, as follows: Group 1 was given distilled water alone; Group 2 was given a single dose of DOX (20 mg/kg); Group 3 was given OMG-3 (100 mg/kg/day); Group 4 was given OMG-3 (200 mg/kg/day), and Group 5 was given OMG-3 (400 mg/kg/day). Groups 3-5 were given OMG-3 orally for four weeks with gavage. Each rat in groups 3-5 received a single intraperitoneal injection of DOX (20 mg/kg) 24 hours after the last doses of OMG-3 to induce acute cardiotoxicity.

Collection of blood samples
Following two days (48 h) of DOX administration, 200 mL of blood was obtained from each rat by heart puncture. The blood samples were placed in an Eppendorf tube containing 600 mL of Triazole reagent, mixed well, and refrigerated at -20°C for molecular analysis.

Measurement of TLR2 and TLR4 gene expression through RT-PCR
The levels of TLR2 and TLR4 gene expression were determined using the real-time polymerase chain reaction (RT-PCR) assay. To determine gene expression fold change data for target genes and the reference gene (β-Actin), the relative quantitative technique 2 (Ct) was used. Amplitude plots of fluorescent signals from each sample vs cycle number showed product accumulation during the RT-PCR. Total RNA was extracted using the Direct-zol TM RNA MiniPrep (Cat. No. R2051, Zymo Research, USA). The reverse transcription (RNA to cDNA) was performed using the Prime Script RT reagent kit (Takara, Japan, Cat. No. RR037A). An aliquot of 2 µL of 5 Prime Script TM was combined with 3 µL total RNA and 10 µL RNase-free dH_2O. The reaction mixture was incubated at 37°C for 15 minutes for the reverse transcription reaction. This was followed by incubation at 85°C for 5 seconds to allow the heat to inactivate the reverse transcriptase. To amplify the reaction, it was then kept at 40°C. All the primers, including target and endogenous genes were 10 pmol. Table 1 shows the nucleotide sequences of the primers. The qPCR assay was performed using the KAPA SYBR® FAST qPCR Master Mix (2X) kit (Sacace, Italy). Each 20 µL of the reaction volume included 5 µl of cDNA, 10 µl of master mix, and 1 µl of both forward and reverse primers, and the reaction volume was completed with nuclease-free water.

Table 1: Nucleotide sequence of primers used for the real-time polymerase chain reaction.

Primer name	Direction	Nucleotide sequence
TLR2	Forward	5′-TATCAGTCCCAAAGTCTAAAGTCG -3′
	Reverse	5 ′- CTACCTCCGACAGTTCCAAGATG-3′
TLR4	Forward	5′-GCCGGAAAGTTATTGTGGTGGT -3′
	Reverse	5 ′- ATGGGTTTTAGGCGCAGAGTTT-3′
Actin	Forward	5′-AGTGCCAGCCTCGTCTCATA -3′
	Reverse	5 ′- GACTGTGCCGTTGAACTTGC-3′

The RT-PCR amplification condition included: enzyme activation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 51°C for 30 seconds, extension at 72°C for 30 seconds, and then final extension at 72°C for 10 min.

S*tatistical analysis*
The statistical analysis system (SAS; 2012) program was used to determine the effect of different factors on study parameters. The least significant difference (LSD) test and analysis of variance (ANOVA) were performed for the mean comparison. A p-value of less than 0.05 was considered a significant difference.

Results and Discussion

The relative quantitative technique 2 (Ct) was used to determine gene expression fold change data for target genes and the reference gene (β-Actin). Amplitude plots of fluorescent signals from each sample against cycle number depict product buildup during the real-time PCR, as shown in Figures 1-3. The mean of the control fold change was utilized as a criterion to evaluate high or low fold expression of TLR2 and TLR4 genes in all samples, including the control, DOX, and OMG-3 groups. The descriptive statistics of TLR2 gene expression (fold change) in the DOX group were significantly ($p \leq 0.01$) elevated (28.74±4.95) compared to the control and the various OMG-3 groups. TLR2 expression was significantly lower in OMG-3 groups (1.633±0.51, 0.733±0.13, and 0.709±0.16) as presented in Table 2 and Figure 4. TLR4 gene expression (fold change) was significantly ($p \leq 0.01$) higher in the DOX group (8.57 ±1.22) compared to the control and OMG-3 groups. Although, there was no difference in fold change among the OMG-3 groups (0.809±0.32, 0.852±0.50, and 0.272±0.16) as shown in Table 3 and Figure 5. The difficulty in treating DOX cardiotoxicity is finding a cardioprotective drug that does not interfere with its anticancer activity.

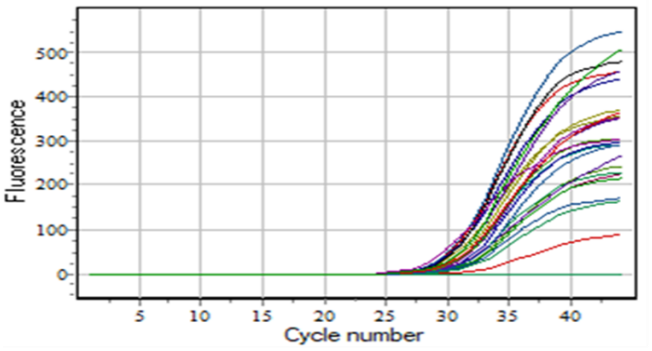

Figure 1: Amplification of TLR2 gene.
The cDNA products of the target gene, and control were subjected to the real-time polymerase chain reaction (RT-PCR). The cycle threshold (Ct) was plotted on the x-axis with the level of fluorescence on the y-axis.

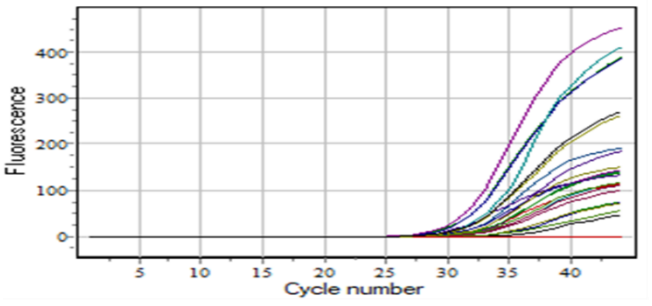

Figure 2: Amplification of TLR4 gene.
The cDNA products of target gene, and control were subjected to the real-time polymerase chain reaction (RT-PCR). The cycle threshold (Ct) was plotted on the x-axis with the level of fluorescence on the y-axis.

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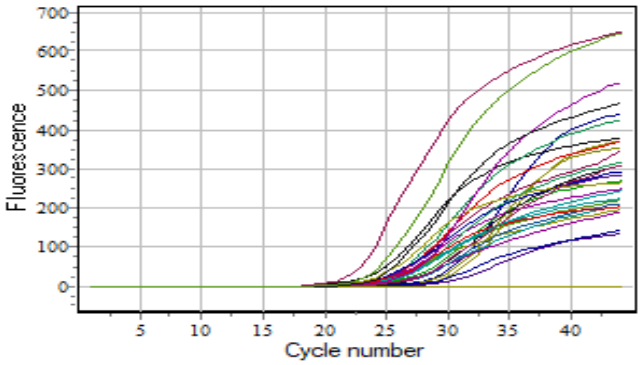

Figure 3: Amplification of reference gene (β-actin).
The cDNA products for all samples were subjected to the real-time polymerase chain reaction (RT-PCR). The cycle threshold (Ct) was plotted on the x-axis with the level of fluorescence on the y-axis.

Omega-3 showed some promising anti-DOX-induced cardiotoxicity properties in this context.[16] Fish and fish oils high in omega-3 fatty acids can help prevent coronary heart disease. Both healthcare providers and the general public are becoming more interested in their roles in the management and prevention of coronary heart disease.[17] As a result, the study was aimed at investigating the cardioprotective effects of OMG-3 in DOX-intoxicated rats, highlighting their capacity to control and inhibit the inflammatory response. In the present study, it was observed that DOX upregulated the expression of TLR2 and TLR4 genes in the cardiomyocytes of rats. It was significantly higher in the DOX group compared to the control and pretreatment OMG-3 groups. This compound is predicted to raise TLR4 and TLR2 levels in macrophages, leading to more intense inflammatory responses to endotoxins and organ damage.[18,19] TLR2 and TLR4 expression (or overexpression) were hypothesized to contribute to the pathogenesis of DOX-induced cardiac dysfunction. Cardiomyocytes express several TLRs, including TLR2 and TLR4. These TLRs allow cardiomyocytes to respond to endogenous or exogenous signals, influencing the pathophysiological responses to dilated cardiomyopathy.[20]
TLRs activate NF-kBp38 (mitogen-activated protein kinase) and Jun N-terminal kinase via many signaling pathways after stimulation. The most common adapter proteins recruited via these routes are the major myeloid differentiation response gene (myD88) and adapter-inducing interferon.[20] Toll-like receptors detect infections linked to specific molecular patterns, such as lipopolysaccharides, peptidoglycan, bacterial lipoproteins, and oligonucleotides, during an inflammatory response.[21] The receptors are critical components of the innate immune system that act in response to pathogens and non-pathogenic causes of injured tissue. There is a relationship between TLRs and the progression of heart failure.[22] TLR signaling inhibition could be a highly effective therapy for DOX-induced cardiotoxicity.[23] TLR2 plays a role in the oxidative stress-induced activation of NF-B in neonatal rat cardiomyocytes. This activation of NF-B is required for cardiomyocyte apoptosis.[24] TLR4 activation not only generates an inflammatory response; but also causes extracellular matrix breakdown, resulting in a vicious cycle that eventually leads to cardiomyopathy.[25] A deficiency of TLR4 can reduce oxidative stress production in the heart and prevent the downregulation of GATA-4, a protein expressed by the GATA4 gene in humans.[26] Previous research has found a link between TLR signaling pathway activation and DOX-induced cardiac injury.[27,28] TLR4 does not have the same strong cardiac selectivity as TLR2, but its presence can aid in the detection of early myocardial dysfunction because TLR4 overexpression has been linked to it. Endogenous signals, such as heat shock proteins and oxidative stress, have been shown in a recent study to activate TLRs and contribute to the development of congestive heart failure. One of the most serious cardiac dysfunctions caused by DOX is the generation of oxidative stress.[29,30] As a result, inhibiting TLR signaling may have a considerable therapeutic effect in the treatment of dilatation cardiomyopathy, particularly when DOX is administered.

Table 2: Gene expression, fold change of TLR-2 in all study groups regarding the effect of omega-3 in doxorubicin-treated rats.

Group	Mean ± SE of TLR-2
Control	1.053 ±0.49[b]
Dox	28.74 ±4.95[a]
100 mg OMG-3	1.633 ±0.51[b]
200 mg OMG-3	0.733 ±0.13[b]
400 mg OMG-3	0.709 ±0.16[b]
LSD	6.808[**]
P-value	0.0001

DOX: doxorubicin; OMG-3: Omega-3; Means with the different letters in the same column differed significantly. [*] (P≤0.05), [**](p<0.01)

Table 3: Mean of gene expression, fold change, of TLR-4 in all study groups regarding the effect of omega-3 on doxorubicin-treated rats.

Group	Mean ± SE of TLR-4
Control	1.231 ±0.51[b]
Dox	8.57 ±1.22[a]
100 mg OMG-3	0.809 ±0.32[b]
200 mg OMG-3	0.852 ±0.50[b]
400 mg Omg.	0.272 ±0.16[b]
LSD	1.957[**]
P-value	0.0001

DOX: doxorubicin; OMG-3: Omega-3; Means with the different letters in the same column differed significantly.
[*] (P≤0.05), [**](p<0.01)

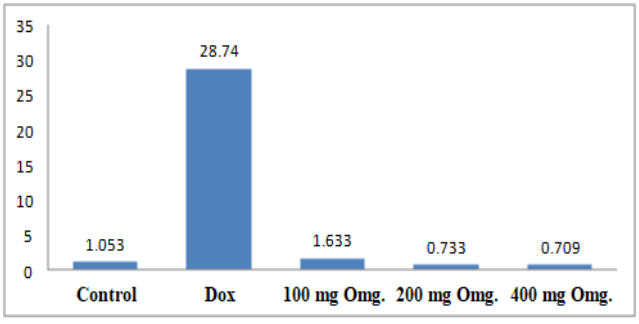

Figure 4: TLR-2 gene expression in different study groups of rats.

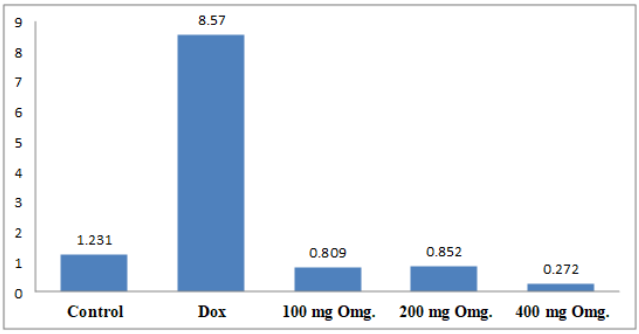

Figure 5: TLR-4 gene expression in different study groups of rats.

Conclusion

The findings of this study reveal that TLR2 and TLR4 fold changes were significantly reduced in the pretreatment OMG-3 rat groups, indicating that OMG-3 may play a role in protecting the heart tissue from DOX treatment.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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