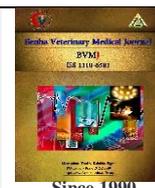




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Immunologic effect of conjugated linoleic acid against doxorubicin-induced toxicity in rats

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ABSTRACT

The current study was carried out to investigate the protective effect of conjugated linoleic acid against doxorubicin-induced toxicity in rats. For this purpose, 40 rats were divided into four groups. control group: rats were injected with a sterile distilled water. Doxorubicin group: rats were injected IP with doxorubicin. Conjugated linoleic acid (CLA) group: rats were received CLA orally. Doxorubicin +CLA group: rats were injected with doxorubicin for 2 weeks then received CLA once daily for 4 weeks. Collection of serum and spleen samples were performed at 4th and 6th week of experiment. Serum was used for evaluation of immunological markers, protein electrophoresis, kidney functions, liver enzymes. Tissue specimens from spleen collected for histopathology examination. The results revealed that there were significant decreases in IgE and IgM, while significant increases in IL-6, urea, creatinine, ALT, AST, ALP were recorded in doxorubicin group. Moreover, histological examination of splenic tissues of doxorubicin group showed congestion of red pulp, lymphoid depletion of white pulp and severe necrosis of lymphoid follicle. Treatment with CLA showed improvement in a forementioned parameters in the first and second check points. It could be concluded that CLA has protective effect against immunosuppression induced by doxorubicin.

1. INTRODUCTION

Doxorubicin (Adriamycin or DOX) is one of the most effective chemotherapeutic agents used in the clinic either alone or in conjunction with other drugs or radiotherapy to treat different types of malignant neoplasia since its isolation in 1960s from *Streptomyces Peucetius* (Sayed-Ahmed et al., 2010). Doxorubicin is considered as immunosuppressive agent due to anti-proliferative and cytotoxic effects, which also determine antitumor effects (Mihich, 2000). Conjugated linoleic acid (CLA) is the common name for a class of fatty acids found in dairy products and ruminant meat (Chin et al., 1992). CLA reduce body fat and enhance glucose metabolism (Turin et al., 2003). Also, CLA inhibit atherosclerotic lesion and increase immune functions (Bassaganya-Riera et al., 2002). Therefore, the aim of this research was to examine the protective effects of conjugated linoleic acid against doxorubicin-induced toxicity in rats via evaluation of hematological, immunological (IL6, IgM, IgE), protein electrophoresis (total protein, albumin, globulin) and biochemical parameters (ALT, AST, ALP), in addition to screening of histopathological changes in spleen.

2. MATERIAL AND METHODS

2.1. Animals

Forty white male albino rats with a weight of 150-170 gm were obtained from the united company of chemicals. All animals were caged and maintained on a standard diet, with free access to tap water. Before beginning the experiment,

the animals were acclimatized for one week. They were kept at room temperature and exposed to a natural light/dark cycle on a regular basis. The current experiment was approved by the committee of Animal Care and Welfare, Faculty of Veterinary Medicine, Benha University, Egypt (BUFVTM 05-06-21).

2.2. Chemical substances

Doxorubicin powder was obtained from RMPL PHARMALLP, Mumbai, India. Conjugated linoleic acid was obtained from Nutrex Research, Inc USA.

2.3. Experimental design

Rats were divided into four groups for this study. Control group: rats were injected with a sterile distilled water. doxorubicin group: rats were injected with doxorubicin (2.5mg/kg, i.p.) every other day for 2 weeks. CLA group: rats received CLA (200mg/kg, p.o.) once daily for 4 weeks from 2nd till 6th week. Doxorubicin+CLA group: Doxorubicin was injected into rats for 2 weeks then received CLA for 4 weeks from 2nd till 6th week (Fouad et al., 2013; Javedan et al., 2016).

2.4. Sampling

Blood samples were taken from the retro-orbital plexus from rats in all groups after 4th and 6th week of the experiment. Samples of blood were taken in clean well-dried centrifuge tubes and by centrifugation at 2500 rpm. for 15 minutes serum was separated, then preserved at -20 °C to estimate biochemical parameters. After collection of blood samples, rats were sacrificed by cervical decapitation. Small tissue specimens were collected and

placed in 10% formalin solution for histopathological investigations.

2.5. Biochemical parameters measurement

Elisa kits were used for quantitative measurement of IL-6, IgM and IgE. They were determined by standard sandwich enzyme immuno-sorbent assay technology described by Ferguson-Smith. (1988). Serum protein electrophoresis was performed by method of Bollag et al. (2002). Colorimetric determination of urea was performed according to Kaplan (1974). Kinetic method for the determination of creatinine was performed according to Fabiny and Ertingshausen, (1971). Kinetic method for determining the activity of AST and ALT were performed according to Schumann and Kluake (2003). Serum ALP was determined using the method described by Sharma et al. (2014).

2.6. Histopathological examinations

Tissue specimens were collected from spleen of rats in all groups then fixed in 10% neutral buffered formalin. The tissues were dehydrated and cleared before being embedded in paraffin and sectioned in 5 µm thick sections. The serial sections were subjected to staining with H&E (Banchroft and Stevens, 1996)

2.7. Statistical analysis

Statistical analysis was performed by SPSS 19.0. Chicago. USA. Differences among the control and treated groups were tested by one-way ANOVA, followed by the Tukey Post-hoc test for multiple comparison. All the values were expressed as mean± S.E. The significance levels were defined as $p < 0.05$.

3. RESULTS

3.1. Immunological parameters

The results of measurement of immunological parameters in Dox injected rats showed significant increase in IL-6 and significant decreases in IgM and IgE levels compared with normal control rats (Tables 1&2). Comparing with Dox group, the estimation of immunological parameters in DOX+ CLA group there were significant decreases in IL-6

and IgE concentration and significant increase in IgM concentration.

Table 1 Changes in immunological parameters in different groups at the 1st check point of the experiment after 4 weeks.

Groups	IL6 (pg/ml)	IgM (ng/dl)	IgE (ng/dl)
Control	4.62±0.17 ^b	108.4±10.73 ^b	216.2±5.99 ^a
Dox	8.24±0.19 ^a	27.00±2.28 ^d	113.00±6.63 ^b
CLA	5.06±0.19 ^b	143.00±5.39 ^a	132.00±3.52 ^b
dox +CLA	4.96±0.23 ^b	65.60±5.89 ^e	87.40±3.57 ^e

a, b & c: There is significant difference ($P < 0.05$) between any two means, within the same column have different superscript letter

Table 2 Changes in immunological parameters in different groups at the 2nd check point of the experiment after 6 weeks.

Groups	IL6 (pg/ml)	IgM (ng/dl)	IgE (ng/dl)
Control	5.18±0.10 ^b	111.40±4.89 ^b	298.00±19.43 ^a
Dox	9.38±0.12 ^a	16.20±2.42 ^d	84.00±12.19 ^e
CLA	5.16±0.22 ^b	170.00±6.52 ^a	114.60±5.40 ^b
DOX +CLA	4.38±0.15 ^c	84.80±4.49 ^c	59.00±9.27 ^d

a, b & c: There is significant difference ($P < 0.05$) between any two means, within the same column have different superscript letter.

3.2. Protein electrophoresis

DOX group comparing with control group, the results of serum protein electrophoresis in DOX group revealed significant of decreases in total protein, albumin, β-globulin (second check point only) and γ-globulin and non-significant changes in α1-globulin, α2-globulin concentrations (Tables 3&4). Comparing with Dox group, the results of serum protein electrophoresis in DOX+CLA group were revealed significant increases in total protein, albumin, α 2-globulin, β-globulin and γ-globulin and non-significant change in α1-globulin concentration in 1st check. while in 2nd check there were significant increases in all protein electrophoresis parameters.

3.3. Biochemical parameters

The results of serum analysis of liver and kidney function testes showed increases in urea, creatinine, ALT, AST and ALP concentration in DOX injected rats compared with non-treated control rats (Tables 5&6). Comparing with control group, the values of these parameters of rats in Dox+CLA group were returned to be nearly like those of control group.

Table 3 Changes in serum protein electrophoresis in different groups at the 1st check point of the experiment after 4 weeks.

Groups	Total protein (g/dl)	Albumin (g/dl)	α1-globulin (g/dl)	α2-globulin (g/dl)	β-globulin (g/dl)	γ-globulin (g/dl)
Control	7.68±0.20ab	4.22±0.09a	0.20±0.03a	0.77±0.06bc	0.80±0.04b	0.93±0.05b
Dox	5.80±0.16c	2.78±0.09b	0.20±0.01a	0.74±0.04c	0.80±0.04b	0.61±0.01c
CLA	7.88±0.18a	4.30±0.07a	0.25±0.02a	0.87±0.03ab	1.04±0.05a	1.22±0.09a
dox + CLA	7.22±0.27b	4.15±0.07a	0.22±0.01a	0.97±0.02a	1.05±0.05a	0.83±0.04b

a, b & c: There is significant difference ($P < 0.05$) between any two means, within the same column have different superscript letter.

Table 4 Changes in serum protein electrophoresis in different groups at the 1st check point of the experiment after

Groups	Total protein (g/dl)	Albumin (g/dl)	α1-globulin (g/dl)	α2-globulin (g/dl)	β-globulin (g/dl)	γ-globulin (g/dl)
Control	7.24±0.20b	4.86±0.10a	0.15±0.02b	0.76±0.02b	0.92±0.02a	0.99±0.03b
Dox	5.38±0.15c	2.38±0.16b	0.15±0.01b	0.75±0.02b	0.75±0.02b	0.57±0.02c
CLA	7.30±0.21b	4.50±0.07a	0.28±0.02a	0.94±0.02a	0.90±0.02a	0.82±0.05b
DOX + CLA	8.38±0.14a	4.78±0.09a	0.26±0.01a	0.94±0.02a	0.96±0.02a	1.23±0.10a

a, b & c: There is significant difference ($P < 0.05$) between any two means, within the same column have different superscript letter.

Table 5 Changes in biochemical parameters in different groups at the 1st check point of the experiment after 4 weeks.

Groups	Urea (mg/dl)	Creatinine (mg/dl)	ALT (U/L)	AST (U/L)	ALP (U/L)
Control	26.60±1.63 ^c	0.90±0.07 ^c	25.20±1.46 ^c	25.60±2.11 ^c	104.40±3.14 ^c
Dox	74.00±0.71 ^a	2.28±0.09 ^a	53.40±1.08 ^a	53.00±2.07 ^a	189.20±2.52 ^a
CLA	27.00±0.71 ^c	1.00±0.07 ^c	26.00±0.71 ^c	26.20±1.52 ^c	106.60±1.00 ^c
DOX + CLA	44.20±1.24 ^b	1.26±0.05 ^b	47.80±0.86 ^b	42.60±2.21 ^b	151.00±1.81 ^b

a, b & c: There is significant difference ($P < 0.05$) between any two means, within the same column have different superscript letter.

Table 6 Changes in biochemical parameters in different groups at the 2nd check point of the experiment after 6 weeks.

Groups	Urea (mg/dl)	Creatinine (mg/dl)	ALT (U/L)	AST (U/L)	ALP (U/L)
Control	23.00±1.14 ^c	1.03±0.05 ^c	24.80±2.03 ^c	27.60±1.81 ^c	89.00±3.52 ^c
Dox	82.20±0.86 ^a	2.79±0.06 ^a	64.80±1.71 ^a	69.00±1.92 ^a	209.00±3.52 ^a
CLA	24.40±0.93 ^c	0.90±0.04 ^c	27.40±1.63 ^c	28.40±1.21 ^c	90.80±1.50 ^c
DOX + CLA	51.00±0.71 ^b	1.54±0.04 ^b	53.00±1.58 ^b	56.40±2.25 ^b	149.20±2.08 ^b

a, b & c: There is significant difference ($P < 0.05$) between any two means, within the same column have the different superscript letter

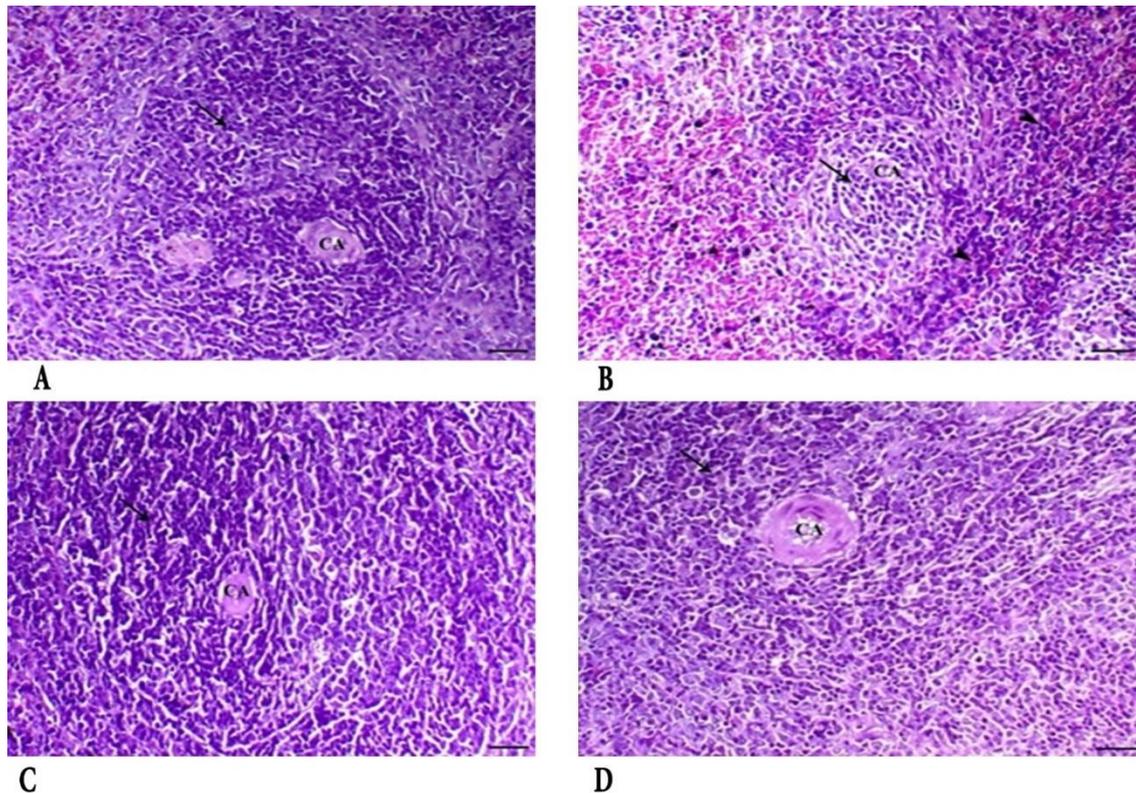


Fig. 1 A) Spleen of rats in control group showing the white pulp contain well defined splenic lymphoid follicle (arrow) with central germinal center and central arteriole (CA), H&E, X200, bar= 100 µm. (B) spleen of rats injected with DOX showing depletion of lymphoid follicle (arrowhead) with very few lymphoid cells (arrow) around the central arteriole (CA), H&E, X200, bar= 100 µm. (C) Spleen of CLA showing dense lymphoid follicle (arrow) around the central arteriole (CA), H&E, X200, bar= 100 µm. (D) Spleen of DOX+ CLA rat showing restoration of splenic architecture and the lymphoid follicles were formed of large number of lymphocytes around the central arterioles H&E, X200, bar= 100 µm.

3.4. Histopathology

The microscopic examination of the spleen of normal rats showed normal white pulp consisted of normal lymphoid follicle around the central arteriole (fig A). The microscopic examination of the spleen of rats injected with DOX alone showed congestion of red pulp and marked lymphoid depletion of the white pulp, severe necrosis of lymphoid follicles with very few lymphoid cells around the central arteriole and lymphoid depletion associated with mild degree of amyloid deposition around the sheath of the follicles of spleen (Fig B). The microscopic examination of the spleen of rats injected with DOX+CLA showed restoration of splenic architecture and the lymphoid follicles were formed of large numbers of lymphocytes around the central arterioles (fig D). CLA group showed mild degree of lymphoid hyperplasia and dense lymphoid follicle around the central arteriole in spleen (fig C).

4. DISCUSSION

In present study, the results of estimation of immunomarkers markers in the DOX injects rats showed significant increase in IL-6 and significant decrease in IgM and IgE when compared with normal control rats. These results agree with Bacharier and Geha (2000), Krishnadasan et al. (2003) and Hiroğlu et al. (2014). The level of IL-6 in the blood correlates strongly with the severity of systemic inflammation and tissue injury, has been linked to delayed apoptosis (Hiroğlu et al., 2014). In phagocytic stimulation, chemokine production, and varied effects on cell growth and death, both TNF- and IL-6 were used (Zhang et al., 2009). Suppression of immunoglobulin production occur after injection of dox to rats as there was significant decrease in IgM and IgE concentration. IgE is controlled by two subtypes of CD4+ T-helper cells (Th1

and Th2). Both Th1 and Th2 have anti-regulatory properties. Th1 cells release interferon- γ like cytokines that suppress cytokines released by Th2 cells, and vice versa. Interleukins such as IL-4, IL-5, IL-6, IL-10, and IL-13 are secreted by Th2 cells. IL-4 and IL13, which are required for B-cell isotype switching to IgE and IL-4 dependent IgE synthesis, respectively. Mast cells are stimulated by IgE when exposed to DOX, Mast cell deprived animals showed greater heart damage (Beer et al., 2016).

Comparing dox + CLA group with dox group showed significant decrease in IL6, IgE concentration and significant increase in IgM concentration. These results agree with Burdge et al. (2004). Because CLA contains a combination of isomers, it reduced tumor necrosis factor (TNF-) and interleukin-6 (IL-6) levels which stimulate immunity in rats and enhance lymphocyte proliferation in broilers. In mice, proliferation of lymphocytes and production of interleukin-2 (IL-2) were both elevated. In animal and human research, these two isomers have differing impacts on certain T cell populations and immunoglobulin subclasses. (O'Shea et al., 2004). CLA increase IgM because of the c9t11 CLA isomer may have a beneficial influence on the proliferative and immunoregulatory response of T cells (Burdge et al., 2004). In addition to decrease in IgE due to CLA has anti-allergic properties. IgE has a central role in type-I (orIgE-mediated) allergic reactions e.g., airborne and food allergens. IgE controls the release of chemical mediators like histamine and leukotrienes by mast cells and basophils, causing allergic reactions. B cells produce IgG1 and IgE antibody isotypes in response to Th2 cells, and CLA controls the balance between Th1 and Th2 responses. (O'Shea et al., 2004).

In this study the result of protein electrophoresis in the DOX injected rats showed significant decrease in total protein, albumin, β -globulin and γ -globulin concentration and non-significant change in $\alpha 1$ -globulin and $\alpha 2$ -globulin, concentration compared with normal control rats. These results agree with Naqshbandi et al. (2012) and Jácevíc et al. (2018). DOX caused decrease in the serum total protein level, particularly albumin content because of DOX-induced nephropathy. Reduced protein intake and utilization, caused in part by appetite loss and extensive lesions in the GIT epithelium, contribute to the development of hypoalbuminemia, edema, weight loss, and generally poor animal health. In animals treated with DOX, the toxic effect on the liver causes inadequate serum protein production. (Herman et al., 2000).

Regarding to DOX injected rats and treated with CLA, it showed significant increase in total protein, albumin, β -globulin, γ -globulin, $\alpha 1$ -globulin, and $\alpha 2$ -globulin concentration when compared with dox group. These findings are consistent with those of Villalpando et al. (2017) who found that CLA possesses antioxidant effects and does not cause hepatotoxicity or statistically affect any renal indicators in rats. CLA group in comparison with control group showed non-significant change in total protein, albumin concentration and significant increase in β -globulin, γ -globulin and $\alpha 1$ -globulin, $\alpha 2$ -globulin. These results agree with Moraes et al. (2012) and Villalpando et al. (2017).

Regarding to kidney function tests, dox group compared with control group showed significant increase in urea and creatinine. These results agree with Mihailović-Stanojević et al. (2009) who mentioned that DOX-treated rats exhibited focal segmental glomerulosclerosis and severe proteinuria. DOX-induced nephrotoxicity is mostly controlled by the selective destruction of proximal tubule cells (Grant et al. 2019). Comparing dox + CLA group with dox group, there was significant decrease in urea and creatinine concentration. These results agree with Schiavon et al. (2019). The CLA diet prevented the change in the renal profile, indicating a protective effect. where CLA, prevented renal cell hyperplasia in a murine model due to metabolic antioxidant properties of CLA (Villalpando et al., 2017). CLA group in comparison with control group showed non-significant change in urea and creatinine concentration. These results agree with Abd El-Gawad et al. (2020).

Concerning to liver function tests, dox injected rats showed significant increase in ALT, AST and ALP compared with normal control rats. These results agree with Jácevíc et al., (2018) who described that DOX was metabolized by liver microsomal enzymes and cytoplasmic reductase to the major metabolite doxorubicin and several hepatotoxic aglycone metabolites. Free radicals and reactive oxygen species (ROS) such as superoxide anions (O_2^-), hydroxyl radicals (OH), and hydrogen peroxide cause lipid peroxidation and protein oxidation, as well as impairing endogenous antioxidant defenses. DOX-induced oxygen radical damage affects hepatic cell membrane lipids, and peroxidation continues autocatalytically, causing structural and functional changes in the hepatic tissue. Hepatocyte apoptosis or necrosis results from irreversible changes, as well as a rise in hepatic enzymes in the blood, particularly ALT, AST, and ALP. When comparing dox + CLA group with dox group there was significant decrease in ALT, AST, and ALP activities. These results agree with Abd El-Gawad et al. (2020) who mentioned that CLA prevent the various cancer-related disturbances caused by an imbalance in the cellular

oxidoreductive status, and the oral administration of CLA-enriched diet led to disappearance of abnormalities in liver functions testes (ALT, AST, and ALP) and returned them to nearly normal levels or improve them as CLA exhibit antioxidant activity. When normal rats received CLA there was non-significant change in ALT, AST and ALP activities compared to normal control rat. These results agree with Mirzaii et al. (2016).

Concerning to histopathological changes, in doxorubicin inject rat when compared with control rat, it showed pathological changes in spleen including congestion of red pulp and marked lymphoid depletion of the white pulp, severe necrosis of lymphoid follicle with very few lymphoid cells around the central arteriole and lymphoid depletion. The present results are in accordance with Halade et al. (2018). Comparing dox + CLA group with dox group revealed restoration of splenic architecture and presence of large number of lymphocytes around the central arterioles in the lymphoid follicles. These results agree with Kelley et al. (2006).

5. CONCLUSION

The results of present study demonstrate protective effects of conjugated linoleic acid against doxorubicin-induced toxicity in rats.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest for current data

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