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Comparison of the Acute and Cumulative Dose Administrations in Doxorubicin-induced Hepatotoxicity via Evaluation of the Histopathological Changes and Inflammation in Rats

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Abstract

Doxorubicin (DOX) may lead to hepatotoxicity when administered chronically or in a high dose. The aim of this study is to determine dose–dependent effects of DOX in rat liver tissue. Thirty male Wistar albino rats were divided into three groups; group I as control, group II as receiving chronically DOX (2 mg/kg, twice in a week, total 20 mg/kg, intraperitoneally) and group III as receiving an acute–single dose of DOX (15 mg/kg, intraperitoneally, on the 20th day) administered groups. At the end of 30th day, animals were sacrificed, and liver tissues were extracted for histopathological and immunohistochemical evaluation. Sections were stained with hematoxylin & eosin to evaluate the histopathological changes and TNF-α and IL-6 expressions were detected by immunohistochemical staining. Both chronic and acute administrations of DOX triggered a significant liver damage. However, it was observed that liver damage induced by acute–single dose DOX administrations were higher than those induced by chronic DOX administrations. TNF-α and IL-6 immunoreactivity was significantly increased in both group II and III group compared to control group. However, immunoreactivity of TNF-α was substantially higher in the group III compared to control. These results demonstrated that acute administrations of DOX relatively induce serious liver damage and inflammatory response.

***Key words**: Doxorubicin, Hepatotoxicity, Inflammation, Liver damage*

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Introduction

Chemotherapy improves the success rate in the cancer treatment but chemotherapeutics generally induces notable toxicity in healthy organs (1). Doxorubicin (DOX) is a chemotherapeutic belonging to anthracycline antibiotics family, and it is used for the treatment of several cancer types (2). The anticancerogenic characteristic of the DOX is referred to its ability to prevent DNA replication by inhibiting DNA and RNA polymerase activity, intercalating with DNA and blocking the movement of the DNA topoisomerase-II enzyme. These events result in apoptosis in most cell types including carcinoma cells(3). However, the administrations of DOX in clinic is restricted because of the harmful side effects on healthy organs such as heart, testis, brain, kidney and liver (4-7).

The mechanism underlying the DOX induced hepatotoxicity have not been clearly elucidated (7), but recent studies have reported the mechanism of doxorubicin hepatotoxicity can be referred cellular apoptosis, lipid peroxidation and oxidative stress (8). Excessive formation and accumulation of the reactive oxygen species (ROS), the activation of the antioxidant defense system and lipid peroxidation of bio-membranes may trigger the overexpression of some factors related to inflammatory pathways and excessive releasing of pro- and anti-inflammatory cytokines such as Tumor Necrosis Factor-alpha (TNF-α) and Interleukin-6 (IL-6). Finally, the unbalance in the formation of these cytokines may cause necrosis and apoptosis. Cytokines are polypeptides expressed and secreted by various cell types and they regulate immune and inflammatory events, including systemic response to inflammation, cell growth, healing and injury (9). Several studies have showed that DOX induce the activation of the inflammatory pathways by causing the upregulation of inflammatory cytokines such as TNF-α and interleukins and cause apoptosis (10, 11). TNF-α stimulates the release of IL-1 and IL-6 into the blood circulation by inducing the mononuclear phagocytes and vascular endothelial cells (12). IL-6 induces the synthesis of many plasma proteins that contribute to the acute phase response such as fibrinogen, hemopexin, cysteine proteinase inhibitor, α_1-anticimotripsin, α_1-macroglobulin by hepatocytes (13, 14).

In cancer chemotherapy, both acute single dose and chronic cumulative doses of DOX are administered to patients with solid tumors or leukemias, but DOX chemotherapy is limited by a cumulative, dose-dependent, and irreversible cardiomyopathy that occurs with repeated administration (15). Moreover, some studies have reported that DOX has a dose-dependent hazardous effects on liver tissue by causing mitochondrial dysfunction in rats (16). Although, the effects of chronic cumulative doses of DOX administrations on heart tissue is widely known, its dose-dependent harmful effects on liver tissue and inflammatory pathways have not been clearly elucidated. The aim of this study is to investigate the dose-dependent effects of DOX on liver tissue via assessment of the changes in the histological structure of liver tissue and the expression levels of inflammatory markers in rats. For this purpose, the detrimental effects of DOX administered acute single dose and chronic cumulative doses in the liver tissue were determined by histopathological examinations and the expression levels of TNF-α and IL-6 were detected via

immunohistochemical staining.

Materials and methods

Compliance with ethical standards

The experimental protocol of this study was approved by the Erciyes University's Experimental Animal and Local Ethics' Committee with number 18/029/2018. 30 male Wistar albino rats (8-12 weeks old, weighing 100-200 gr) were obtained from Hakan Çetinsaya Experimental and Clinic Research Center, Erciyes University, Kayseri, Turkey. Rats were hosted in a 12 h light/12 h dark cycle at room temperature (20-24 °C) and environmental humidity during the experimental period and they were fed with standard diet ad libitum.

Chemicals

Doxorubicin was purchased from Koçak Farma, brand name of which is Adrimisin. DOX Koçak 50 mg iv/1 flacon + 1 amp, containing lyophilized powder for intravesical infusion, was used.

Experimental design

Animals were divided into 3 groups as follows; the Control group (n=10) untreated rats, the Chronic DOX group (n=10) injected 2 mg/kg/twice in a week/cumulative 20 mg/kg DOX intraperitoneally (i.p.) similar to the literature (17), and the Acute DOX group (n=10) group given 15 mg/kg (18) DOX i.p. at the 1st day of the experiment and sacrificed on the 10th day of the experimental period. After application of the procedure, animals were sacrificed under the ketamine (30 mg/kg) and xylazine (4 mg/kg) anesthesia. After sacrifice, liver tissues were extracted for the histopathological and immunohistochemical analyses.

Histopathological evaluation

Histopathological evaluation of the liver was performed according to routine histological methods. Samples were fixed in 10% formaldehyde for 24–48 h, dehydrated with alcohol series, cleared with xylene and embedded in paraffin blocks. Then, they were cut into 5-µm thick sections. Hematoxylin-eosin (H&E) staining was performed for determining the histopathological changes in the liver tissue. Histopathological changes in the liver tissue sections were evaluated by the study group at 50 random microscopic area for each experimental group (19, 20). Images were obtained with a light microscope (Olympus BX51; Olympus, Tokyo, Japan).

Immunohistochemistry

The immunohistochemical staining was applied according to protocols used in literature (21, 22) to determine the changes in the immunoreactivities of TNF-α and IL-6 antibodies in the liver tissue. 5 µm sections were cut from the paraffin blocks and they were stored in the oven at 60 °C for 2 hours for the removal of the excessive paraffin. The tissues were treated with xylene for the deparaffinization and they were hydrated with alcohol series. Sections were taken into a sterile urine container with 0.01 M citrate buffer and heated in a microwave oven at 350 W for the antigen retrieval. After washing with the phosphate-buffered saline (PBS) for 5 minutes at three times, sections were treated with 3% (w/v) H_2O_2 for 10 minutes to inhibit the endogenous peroxidase activity. After rewashing three times with PBS, Ultra V Block solution was applied to the sections and they were stored in the incubation tank for 5 minutes. After then, TNF-α (Cat. No: E-AB-52065, Elapscience, USA) and IL-6 (Cat. No: E-AB-40073, Elapscience, USA) antibodies diluted in the ratio of 1:100 were added to the tissues and incubated overnight at 4 °C.

On the following morning, sections were rewashed 3 times with PBS, and the secondary antibody (TA-125-HDX, Thermo Fisher Scientific, Waltham, MA, USA) was applied to sections for 10 minutes. After rewashing with PBS, the immunoreaction was amplified by using the streptavidin–avidin–peroxidase solution, and 3,3-p-diaminobenzidine tetrahydrochloride (TA-060-HDX, Thermo Fisher Scientific, Waltham, MA, USA) was used for the visualization of the immunoreaction. Then, Gill hematoxylin was used for the counter-staining of the sections. At final stage, alcohol series were used to dehydration of the liver tissues, they were passed through xylene, and finally, they were covered up by entellan. Photographs were taken with a light microscope. In order to measure the density of immunoreactivities on the images, Image J (1.45s, National Institute of Health, USA, RRID: SCR 003070) program was used and obtained measurements data were analyzed by the study group according to previous studies (23-26).

Statistical analysis

All statistical analyses were carried out by using GraphPad Prism version 7.00 for Mac, GraphPad Software, La Jolla, California, USA. D'Agostino Pearson omnibus test was used to identify the normal distribution of the data. In the case of normal distribution, quantitative variables were compared using one-way analysis of variance (ANOVA) and Tukey's post–hoc test. Kruskal Wallis and Tukey's post–hoc tests were used for comparing the quantitative with the abnormal distribution. The data were expressed as the mean of normalized data ± standard deviation of the mean. $p<0.05$ was considered as statistically significant.

Results

Doxorubicin induce a significant liver damage

All liver sections of Control group showed normal histomorphology. In the Chronic DOX group, sinusoidal dilatation and intracytoplasmic vacuoles were observed in many sections. Moreover, there was no lipid accumulation in the Chronic DOX group. The radial hepatocyte appendages that begin in the vena cava were damaged (Figure 1B). However, in the Acute DOX group radial hepatocyte appendages were relatively more irregular than those in the Chronic DOX group. Moreover, in addition to sinusoidal dilatation and intracytoplasmic vacuoles, eosinophilic necrotic cells and hemorrhage were observed in the liver sections of Acute DOX group. Similar to Chronic DOX group, lipid accumulation was not observed in the Acute DOX (Figure 1C).

Both Acute and Chronic administrations of DOX increased the expressions of TNF-α and IL-6

Immunohistochemical staining was performed using the avidin-biotin method to determine the liver tissue expressions of TNF-α and IL-6. Immunohistochemical examinations demonstrated the presence of TNF-α and IL-6 immunostaining in the lobules of liver tissue. The TNF-α and IL-6 expressions in the liver of Control group showed weak immunoreactivity (Figure 2A, 2D). TNF-α immunoreactivity significantly increased in the liver tissues of both Chronic DOX group ($p<0.0001$) and Acute DOX group ($p<0.0001$) when compared with the Control group. Moreover, immunoreactivity of TNF-α was substantially higher in the Acute DOX group compared to Chronic DOX group ($p=0.0249$) (Figure 2B, 2C) (Figure 3A).

IL-6 immunoreactivity considerably increased in both Chronic DOX (p<0.0001) group and Acute DOX group (p<0.0001) when compared with the Control group. However, there was no significant difference between immunoreactivity levels of IL-6 in the Chronic DOX and Acute DOX groups when compared each other (p=0.236) (Figure 2E, 2F) (Figure 3B). Immunoreactivity density measurements obtained via Image J program and statistical analysis of the measurements were given in Table 1 and Figure 3.

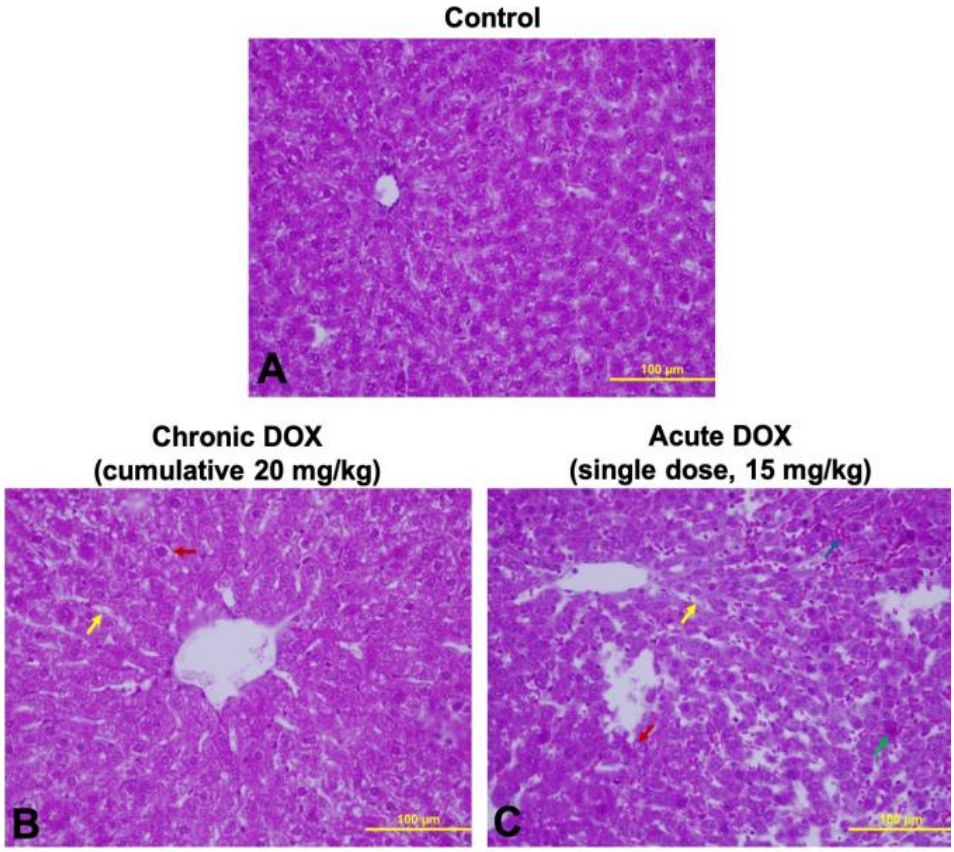

Figure 1: Light microscopy of liver tissue stained with H&E; **A**, Control group (n=10); **B**, Chronic DOX group (n=10) administered cumulative 20 mg/kg doxorubicin; **C**, Acute DOX group (n=10) administered single dose 15 mg/kg doxorubicin. Yellow arrows show the sinusoidal dilatation. Red arrows show the intracytoplasmic vacuoles. Green arrow shows the eosinophilic necrotic hepatocytes. Blue arrow shows hemorrhage. Scale bar=100 µm. Abbreviations: H&E, hematoxylin-eosin; DOX, doxorubicin.

Table 1: TNF-α and IL-6 immunoreactivity measurements of experimental groups. All data are expressed as the mean ± standard deviation (n=10). Abbreviations: DOX; doxorubicin, TNF-α; Tumor Necrosis Factor-α, IL-6; Interleukin-6.

Group	Control	Chronic DOX	Acute DOX	*p*
TNF-α	95.80 ± 4.20	112.38 ± 11.00[a]	117.16 ± 7.11[b]	**0.001**
IL-6	96.91 ± 7.87	108.55 ± 8.86[a]	110.95 ± 10.09[a]	**0.001**

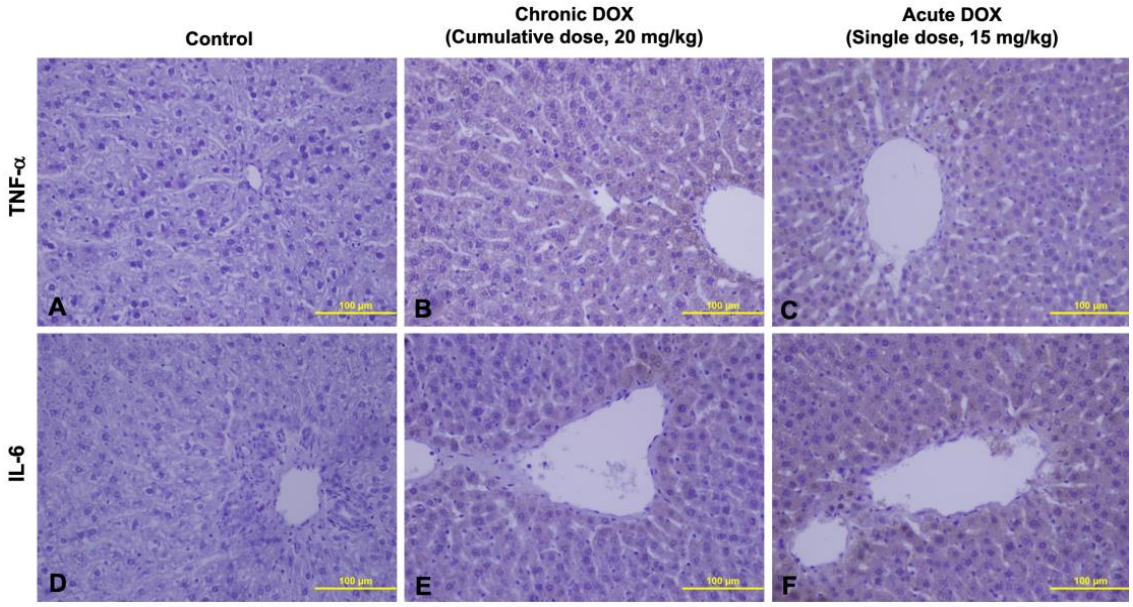

Figure 2: TNF-α and IL-6 immunostaining of liver tissues in experimental groups. **A, D**: Control group (n=10) show weak TNF-a and IL-6 immunoreactivity in liver sections; **(B, E)** Chronic DOX group (n=10) and **(C, F)** Acute DOX group (n=10) shows strong TNF-α and IL-6 immunoreactivity when compared to Control group. Abbreviations: DOX; doxorubicin, TNF-α; Tumor Necrosis Factor-α, IL-6; Interleukin-6.

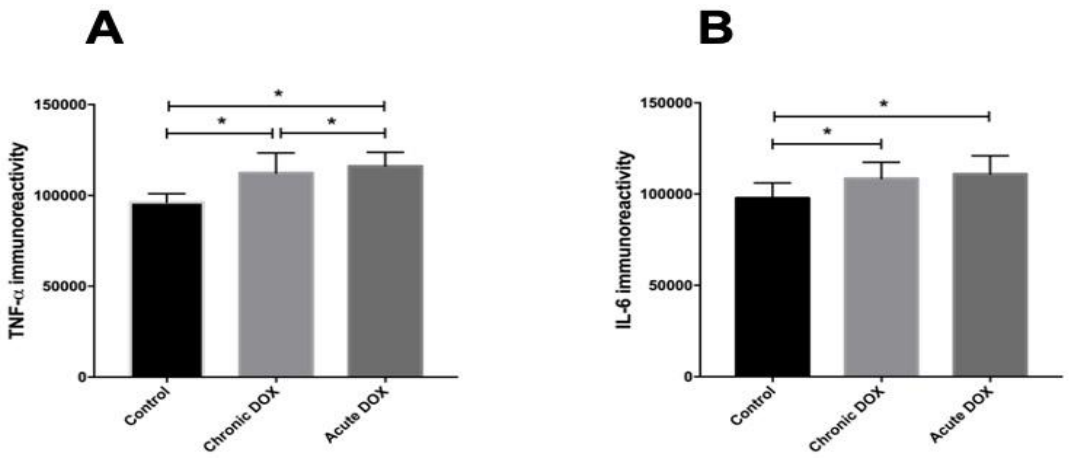

Figure 3: Statistical analysis of the immunoreactivity density results obtained via Image J software. Graphs show the changes in the immunoreactivities of TNF-α and IL-6 among experimental groups. **A**, TNF-α immunoreactivity significantly increased in Acute DOX group when compared to Control and Chronic DOX groups. **B**, IL-6 immunoreactivity substantially increased in Acute and Chronic DOX groups when compared to Control group. * shows statistical significance when p <0.05 among experimental groups.

Discussion

Liver regulates many vital processes such as defense mechanism of the body, nutrient processing, lipid and protein metabolism, glycogen storage, blood filtration, removal of bilirubin, bile production, urea synthesis, and detoxification of drugs and toxic substances. Because of these functions, the liver continually exposes to environmental pollution, toxic chemicals, and chemotherapeutic agents (27). Liver has a high regeneration ability to tolerate

the detrimental effects of toxic agents. However, the normal morphology of the liver begins to get damaged as the harmful agents accumulate and trigger some cytoplasmic pathways (28).

Chemotherapy is an important method for the treatment of many cancer types but chemotherapeutics generally induces significant toxicity (1). Although DOX is used for treatment of many cancer types and it has a potent antineoplastic activity, clinical administrations of DOX cause toxic effects such as cardiotoxicity (29), nephrotoxicity (30), hepatotoxicity (7) and gonadal toxicity (31). Both acute single dose and chronic cumulative doses of DOX are administered to patients with solid tumors or leukemias, but DOX chemotherapy is limited by a cumulative, dose-dependent, and irreversible cardiomyopathy that occurs with repeated administration (15). Moreover, recent studies have reported that dose-dependent DOX administrations induce a significant liver damage by causing changes in the expression levels of Nrf1, Pink1, HSP70, and Sirt3, which are playing a key role in mitochondrial biogenesis, mitophagy, and mitochondrial protein acetylation respectively (16). However, the dose-dependent effects of DOX on the liver tissue and the comparison of the effects of acute single dose and cumulative chronic dose administrations of DOX on the liver inflammation have not been clearly revealed. In this experimental study, we compared the acute single dose and cumulative chronic dose administration of DOX in liver tissue of Wistar albino rats. According to our histopathological examinations, DOX induced a significant liver damage in both acute single doses and cumulative chronic doses. However, in the Acute DOX group administered 15 mg/kg single dose of DOX showed more histopathological changes in liver tissue, including eosinophilic necrotic hepatocytes and hemorrhage in addition to sinusoidal dilatation and intracytoplasmic vacuoles that were observed in the Chronic DOX group when compared with Chronic DOX group, administered 20 mg/kg cumulative doses of DOX. We think that the liver damage in the Chronic DOX group was relatively lower than those in the Acute DOX group is due to high regeneration ability of the liver to tolerate the detrimental effects of toxic chemotherapeutics. Thus, we suggest that the acute high dose administrations of the DOX is more dangerous for the liver tissue morphology and its function.

In order to activation of the systemic response to inflammation, most cell types express and secrete particular polypeptides which are called pro- and anti-inflammatory cytokines (9). Several studies have suggested that DOX activates the inflammatory pathways by inducing the overexpression of the inflammatory cytokines such as TNF-α and IL-6 and drives cells to apoptosis in the liver tissue (32). Our immunohistochemistry result showed that DOX triggered a significant inflammation in the liver tissue by inducing the upregulation of TNF-α and IL-6 in the DOX administered groups when compared to Control group. Immunoreactivity of these cytokines was significantly increased in both acute 15 mg/kg single dose administered DOX group and cumulative 20 mg/kg administered DOX group. Moreover, there was also a significant difference between Acute DOX and Chronic DOX group in terms of TNF-α immunoreactivity. Because it is known that TNF-α is one of the cytokines that make up the acute phase

reaction of inflammation (33), we think that the expression of TNF-α significantly increased in Acute DOX group when compared to Chronic DOX group due to this function of TNF-α. According to our immunohistochemical data, we suggest that the inflammation level induced by acute single dose administrations of DOX is higher than those in cumulative chronic administrations. Thus, increased inflammation levels may result in activation of apoptotic pathways in the liver tissue, suggesting more tissue damage affecting the normal function of the liver negatively.

Conclusion

In this study, we compared the detrimental effects of the acute single dose and cumulative chronic dose administrations of the DOX on liver tissue by determining the histopathological changes and the changes in the expression levels of TNF-α and IL-6 that trigger the inflammatory response. In the light of our histological and immunohistochemical examinations, we suggest that the acute single dose administrations of DOX have more hazardous effects in the liver tissue in terms of histopathological changes and inflammation compared to cumulative chronic administrations. Our results may contribute to the dosing of the acute single-dose administrations of the chemotherapeutics exerting similar effects with DOX and consideration of the anti-inflammatory agents as a protective therapy before the administration of single doses in the treatment of many cancer types.

Conflict of interests

The authors declare no conflict of interests.

Acknowledgement

All researches attributed equally to the study.

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