

Effect of N-Acetyl Cysteine on Doxorubicin Induced Cardiotoxicity in Adult Male Albino Rat: Histological Study

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Abstract

Introduction: Cardiotoxicity is one of the most important causes that limit the use of Doxorubicin (DOX) in treatment of the cancer. N-acetylcysteine (NAC) is an antioxidant substance that protects different cellular organelles from free radicals.

The aim of the work: To shed the spot on the role of NAC on cardiotoxicity induced by DOX.

Materials and methods: Forty adult male albino rats were divided into three groups. Group I: it was formed of 20 animals served as a control group. Further, it was divided into two subgroups; Subgroup Ia: formed of 10 animals received physiologic saline and Subgroup Ib: formed of 10 animals received NAC. Group II: formed of 10 animals that received DOX dissolved in normal saline. Group III: formed of 10 animals that received DOX similar to group Ib. At the end of the second week, all animals were sacrificed; the heart specimens were dissected out and processed to light and electron microscopic examination. Morphometric and statistical analysis was also done.

Results: Light microscopic examination of group II showed deeply stained cardiac muscle fibers and congested coronary vessels. Distorted cardiac muscle fibers and deeply stained nuclei were also observed. Moreover, cellular infiltration was also observed among cardiac muscle fibers. Apparent increase in greenish collagen fibers was seen between cardiac muscle fibers by Masson's trichrome. Electron microscopic examination of group II showed the cardiac muscle with thinning out of some myofibrils, vacuolations of the sarcoplasm and irregular wavy nuclear envelop. Telocytes appeared between cardiac muscle fibers. Group III showed improvement of the cardiac muscle by light and electron microscope with minimal vacuolation in the cardiac muscle. Morphometric and statistical analysis confirmed the histological results.

Conclusion: The present study demonstrated that the administration of N-acetyl cysteine could protect against cardiaotoxicity induced by doxorubicin.

Keywords: Cardiac muscle; Doxorubicin; N acety cysteine; Telocytes; Rats

Introduction

Doxorubicin (DOX) is a common antineoplastic anthracycline antibiotic and is used for treatment of many types of cancer [1]. However, the risk of cardiac, renal, pulmonary, testicular, and hematological toxicities largely limits its effective and widespread use in clinical oncology [2]. The risk of developing cardiac impairment increases concomitantly with an increase in the cumulative dose [3]. In a recent study based on endomyocardial biopsies, it has been found that cardiac muscle damage begins before clinical signs become evident [4].

The heart is more susceptible to DOX-induced lipid peroxidation and toxicity because of its high energy requirement and high mitochondrial density. Further, the heart also lacks the antioxidant enzymes needed to detoxify superoxide anions and hydrogen peroxide; thus, the generated free radicals accumulate and cause severe lipid peroxidation, leading to extensive destruction of the cardiac cellular mitochondrial membranes, endoplasmic reticulum, and nucleic acid [1,5]. The pathophysiological background of DOX cardiotoxic effect is multifactorial and not completely elucidated [5,6]. This damage produced by DOX is dose-related and may lead to cardiomyopathy [7,8].

N-acetyl cysteine (NAC) is an antioxidant substance that plays an important role in the protection of cell constituents from oxidative stress [9].The standard treatment for DOX induced systolic heart failure is with angiotensin-converting enzyme inhibitors. Few trials have suggested that N-acetyl cysteine (NAC) may reduce the incidence of left ventricular dysfunction in high risk patients after DOX chemotherapy [10].

Aim of the Work

This study was aimed to assess the effects of DOX on male rat cardiac muscle and the possible role of NAC.

Materials and Methods

Forty Wister adult male albino rats with average weight 100 - 150 gm were used in this study. The animals were kept in the scientific research center, Faculty of Medicine, Ain Shams University for one week for acclimatization. The animals were randomly divided into three groups. Each group was kept in galvanized wired cage through the duration of experiment. All the experimental procedures were carried out according to the recommendation and the guidelines of the institutional animal ethics committee at Faculty of Medicine, Ain Shams University.

Group I: it was formed of 20 animals served as control group. Further it was divided into two subgroups; subgroup Ia and Subgroup Ib.

Subgroup Ia: it was formed of 10 animals received intraperitoneal injection of 0.5ml of 0.9% physiologic saline three times a week for 2 weeks.

Subgroup Ib: it was formed of 10 animals received NAC by nasogastric at a dose of 250 mg/Kg/ day for 2 weeks [11].

Group II: it was formed of 10 animals that received DOX dissolved in 0.5 ml 0.9% physiologic saline intraperitoneally at a dose of 2.5 mg/Kg/ three times a week (Sunday, Tuesday and Thursday) for 2 weeks (cumulative dose: 15 mg/kg) [12].

Group III: it was formed of 10 animals that received DOX similar to the group II and NAC by nasogastric at a dose of 250 mg/Kg/ day for 2 weeks [11].

Doxorubicin was purchased in the form of vials 50mg/25ml. It is manufactured by EBEWE Pharma Ges.m.b.H. Nfg.KG-Austria.

N-acetyl cysteine was purchased in the form of sachets: 600 mg/ sachet. It is manufactured by Sedico Egypt.

All animals were kept in standard conditions, well aerated room and exposed to 12 hour dark /light cycle. They were freely allowed to water and food ad libitum.

At the end of the second week, all animals were sacrificed and the heart specimens were dissected out and divided into two ways of processing.

For light microscopic study

The specimens were prepared as usual and paraffin sections at 7-9 μ m were cut [13]. The following histological stains were done:

- H & E stain.
- Masson's trichrome.

All sections were stained and prepared for examinations.

For electron microscopic study

Specimens were taken from the wall of left ventricle at a size of 1mm3. They were fixed in glutraldehyde, then in osmic acid, dehydrated in ascending grades of alcohol, cleared in propylene and embedded in resin. Semithin sections were cut for selection of the proper field for ultrathin sections. Ultrathin sections were stained by urenyl citrate and prepared for electron microscopic examinations. Specimens were examined and photographed using a JEM 1200 EXII transmission electron microscope, JEOL, Tokyo, Japan (TEM) at the Faculty of Science, Ain Shams University [14].

Morphometric and statistical study

- The mean thickness of cardiac muscle fibers was measured in 5 non-overlapping fields (magnification X400) in 5 different sections in H & E stained sections.
- The mean area percentage of collagen among cardiac muscle fibers was measured in 5 non-overlapping fields (magnification X400) in 5 different sections in Masson's trichrome stained sections.

These data were calculated by image analysis program (Leica, QwinQgo)(Microsystems Imaging Solutions Ltd, Cambridge, UK) in Histology Department, Faculty Of Medicine Ain Shams University.

The data were expressed as mean and standard deviation. Student's "t" test was used to compare the morphometric data and the P value was calculated using SPSS program (version 17; SPSS Inc., Chicago, Illinois, USA). Statistical significance was determined at a level of P < 0.05. So, P > 0.05 was considered non-significant and P value <0.05 was considered significant (n = 6)[15].

Results

Light microscopic results

Both subgroups Ia and Ib showed similar results by light and electron microscope. These were also confirmed statistically.

Light microscopic results: by examination of H & E stained sections the cardiac muscle fibers were arranged in branching and anastomosing manner. The fibers were separated by connective tissue that contains the coronary blood vessels (Figure 1). Each cardiac muscle fibers showed acidophilic sarcoplasm and transverse striations. It also contained centrally vesicular oval nucleus. The connective tissue between the fibers had many fibroblasts with elongated nucleus (Figure 2).





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Figure 2: The cardiac myocytes with acidophilic striations (\uparrow) and centrally located oval vesicular nucleus (N). Notice the elongated nuclei of the fibroblasts (F) in the connective tissue between the cardiac muscles. Group I. H and E x400.



Figure 3: Cardiac myocytes with some deeply stained sarcoplasm (D) in some areas and apparently congested coronary vessels (†). Group II. H and E x100.

In group II examination of H & E stained sections; the cardiac muscle fibers showed deeply stained sarcoplasm and congested coronary vessels in between the muscle fibers (Figure 3). Moreover, disrupted and destructed cardiac muscle fibers were seen and deeply stained nuclei were observed in other fibers together with extravasated blood (Figure 4). Cardiac muscle fibers showed alternating deeply stained and pale stained sarcoplasm, loss of myofibers and deeply stained pyknotic nuclei were also observed (Figure 5,6). Moreover, cellular infiltration in between the cardiomyocytes and around a blood vessel was seen (Figure 7). In H & E stained sections of group III, the cardiac muscle fibers appeared in branching and anastomosing arrangement separated by connective tissue containing blood vessels (Figure 8). The cardiac myocytes showed acidophilic striations and oval vesicular centrally located nuclei (Figure 9).



Figure 4: Distorted destructed cardiac myocytes (D) and deeply stained nuclei (\uparrow). Notice the extravasated blood (E). Group II. H and E x400.

By Masson's trichrome stained sections the greenish collagen fibers were apparently few and minimum among the cardiac muscle fibers in group I(Figure 10) while in group II an apparent increase in collagen was seen between cardiac muscle fibers (Figure 11). Moreover, an apparent decrease in greenish collagen fibers appeared between the cardiac myocytes in group III (Figure 12).



Figure 5: Cardiac myocytes with deeply acidophilic homogenous sarcoplasm (\uparrow) and loss of striations. Group II. H and E x400.

Electron microscopic results

The cardiac myocytes of control group I showed myofibrils, numerous mitochondria and central euchromatic nuclei. The myofibrils consisted of actin and myosin that showed alternating dark and light bands. The myofibrils were formed of repeated successive sarcomeres each one formed of the distance between two adjacent Z lines. H zone and M line bisected it were found at the middle. Numerous mitochondria were distributed between the myofibrils. Diad systems of cardiac muscle fibers were noticed at the Z lines of sarcomeres (Figure 13). Intercalated disc was located between adjacent cardiac muscle fibers. It showed step like structure with many

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desmosomes and fascia adherents. Less electron dense regions of the disc had abundant gap junctions (Figure 14).



Figure 6: Widely separated muscle fibers and alternating deeply stained (D) and pale stained (P) sarcoplasm. Notice the areas of myofibers loss (\blacktriangle) and deeply stained pyknotic nuclei (\uparrow). Group II. H and E x400.





Electron microscopic examination of group II showed the cardiac muscle fibers with thinning out of some myofibrils and vacuolations of the sarcoplasm. The vacuolations were distributed among the myofibrils and more around the centrally located nuclei. Moreover, the cardiomyocytes showed irregular wavy nuclear envelop around the nucleus (Figure 15). Telocytes appeared in the interstitium in close relation to the vacuolated cardiac muscle fibers and disrupted intercalated disc was also observed (Figure 16). Telocytes sent their processes (telopodes) in close relation to both the sarcolemma of cardiac muscle fibers and blood vessels (Figure 17). Many cells with euchromatic nucleus and others with myofibrils were seen in between the cardiac myocytes (Figure 18).



Figure 8: Branching and anastomosing cardiac muscle fibers nearly similar to the control (\uparrow) . Group III. H and E x100.



Figure 9: The striations of cardiac myocytes (\uparrow) and centrally located oval vesicular nucleus (N). Notice the fibroblasts (F) in between the cardiac muscles. Group III. H and E x400.

Group III by electron microscopic showed the cardiac muscle with numerous mitochondria and myofibrils (Figure 19). The intercalated disc was nearly similar to that of the control (Figure 20).

Morphometric and statistical results

The mean thickness of cardiac muscle fibers of subgroup Ia was 12.8 \pm 0.86 µm. Subgroup Ib expressed no statistically significant difference compared with subgroup Ia. There was significant increase in group II in comparison to subgroup Ia, Ib and group III. There was no significant change in the mean thickness of cardiac muscle fibers group III compared with subgroup Ia and Ib (Table 1 and Figure 21,).

The mean area percentage of collagen by Masson's trichrome stain of subgroup Ia was 4.7 \pm 0.57. Subgroup Ib expressed no statistically significant difference compared with subgroup Ia. There was significant increase in group II in comparison to subgroup Ia, Ib and group III. There was no significant change in group III compared with subgroup Ia and Ib (Table 1 and Figure 22).

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Figure 10: The connective tissue among the cardiac muscles with apparently minimal content of greenish collagen fibers (*↑*). Group I. Masson's trichrome x400.



Figure 12: The connective tissue among the cardiac muscles with apparently few greenish collagen fibers (\uparrow). Group III. Masson's trichrome x400.



Figure 11: The connective tissue among the cardiac muscles with apparently abundant greenish collagen fibers (\uparrow). Group II. Masson's trichrome x400.



Figure 13: A cardiac myocyte with its euchromatic nucleus (N). The sarcoplasm filled with regularly spaced myofibrils (MF) and numerous elongated mitochondria with apparent cristae (mit). Notice the Z lines (Z) and diad system (D). Group I. TEMx6000.









Figure 16: The telocyte with indented euchromatic nucleus and multiple processes between cardiac myocytes (\uparrow). Notice the vacuolation (V) within the sarcoplasm of cardiac myocytes and disrupted intercalated disc (ID). Group II. TEMx2000.



Figure 15: Vacualation (V) within the sarcoplasm of cardiomyocyte. The nucleus shows irregular wavy nuclear envelop (†). Notice the irregularly arranged myofibrils (MF). Group II. TEMx3000.



Figure 17: Tortuous processes (\uparrow) of telocytes in close relation to cardiac muscle fibers (CMF) and blood vessel (BV). The dilated end of the process contains mitochondrion (\blacktriangle). Group II. TEMx6000.







Figure 22: Percentage of collagen stained by Masson's trichrome in different groups.

Groups	Mean thickness of cardiac muscle fibers in μm Mean ± SD	Mean area percentage of collagen by Masson's trichrome stain Mean ± SD
Subgroup la	12.8 ± 0.86	4.7 ± 0.57
Subgroup Ib	12.65 ± 0.95	4.8 ± 0.29
Group II	17.6 ± 0.8*	11.58 ± 1.03 *
Group III	13.1 ± 0.91#	5.36 ± 0.79#

*Significant increase P value for t test< 0.05in group II compared with other groups.

#non-significant change P value for t test > 0.05 in group III compared with subgroup Ia and Ib.

Table 1: The mean thickness of cardiac muscle fibers in μ m in H & E stained sections and means area percentage of collagen fibers in Masson's trichrome stained sections in different groups.

Discussion

In spite of being an effective anti-cancer agent, DOX usage at maximal therapeutic dose is life threatening due to its accumulation in the circulation which may induce irreversible cardiomyopathy and heart failure [16]. Following DOX treatments, aberrations to myocardial architecture and function include cardiac cell hypertrophy and death, heightened susceptibility to myocardial infarction (MI), cardiomyopathy, and left ventricular dysfunction [17-19].

There are few reported studies about N-acetyl cysteine NAC on non-ischemic cardiomyopathy, such as DOX-induced cardiomyopathy and heart failure. Our study provides the important evidence of the beneficial effects of NAC on cardiac dysfunction resulting from DOXinduced heart injury in terms of myocardial fibrosis.

In group II the animals received DOX, the cardiac muscle fibers showed deeply stained sarcoplasm and vacuolated in other fibers areas. This could be explained by some authors who reported that free radicals created by DOX with the mitochondrial and endoplasmic reticulum membranes [20]. Moreover, cellular infiltration was observed around the blood vessels of connective tissue and between cardiac muscle fibers. Cardiac muscle swelling also was confirmed by morphometric and statistical analysis. These could be explained by the oxidative stress produced by DOX that leaded to release of cytokines attract inflammatory cells and vasodilate the vessels. These were in accordance to some researchers who explained that one of the features of DOX is the induction of apoptosis of both terminally differentiated cardiomyocytes and cardiac progenitor cells, resulting in the loss of myocardial tissue and intrinsic regenerative capacity, respectively [21,22].Other authors [23], said that doxorubicin impairs the insulin-like growthfactor-1 system and causes insulin-like growth factor-1 resistance in cardiomyocytes.

In our study fibrosis was observed in DOX induced cadiomyopathy. The fibrosis was confirmed by morphometric and statistical analysis. These findings were explained by many authors [24]. They recorded that TGF- β 1 is a protein secreted by cardiac myofibroblast and fibroblast that controls proliferation and is responsible for cardiac muscle hypertrophy, and interstitial fibrosis. Previous reports showed that TGF- β 1 gene expression is increased in the left ventricular myocardium of patients with idiopathic hypertrophic cardiad infarction [25]. Fibrosis plays a major role in adverse cardiac remodeling in DOX induced cardiomyopathy and post myocardial infarction [26]. These findings suggest the possible involvement of TGF- β 1 gene and protein in the regulation of DOX cardiac fibrosis. The inflammatory response caused by injury initiated the processes of tissue repair which could cause tissue fibrosis.

Mitochondrial damage and thinning out of myofibrils in the same group (DOX treated group). These went side by side with many authors[27,28] who stated that oxidative stress of membranous organelles especially mitochondria lead to ATP depletion and more consumption of glycogen to maintain the homeostasis of cardiac muscle fibers.

In the present study telocytes were demonstrated in DOX treated group. This was in accordance with many authors. They reported the role of telocytes in cardiac tissue regeneration and repair [29,30]. They added that stem cell-based tissue regeneration therapy is thought to be a potential and promising method to treat some diseases in different tissues and organs, such as the heart. Recently, other authors [31] confirmed the growing evidences that there is a close contact between telocytes, stem cells and progenitor cells in stem cell niches in the heart. Others [32] explained that the 3D interstitial network constructed by telocytes not only provided a mechanical support for stem cells and progenitor cells but also promoted proliferation, differentiation, maturation, and migration of stem cells. They explained that the main mechanisms of interaction between these cells are atypical junctions and paracrine effects.

In group III the animals received both DOX and NAC for 2 weeks, the cardiac muscle showed improvement. There were improvement in vacuolations and fibrosis. Moreover, the glycogen content appeared similar to that of the control. The cardiac muscle showed picture similar to that of the control. Thus, the use of substances with antioxidant properties has been proven effective at protecting the cardiac muscle against damage caused by reactive oxygen species (ROS) generation. In this sense, several studies have shown that NAC can attenuate the effects caused by DOX [33]. They reported that NAC was able to restore the anti- and prooxidant balance of the cardiac muscle and prevent the harmful effects of DOX treatment. Morphometric and statistical analysis confirmed the histological picture of NAC against DOX as regard the fibrosis and swelling. These were in accordance to many authors [34,35]. They reported that NAC

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is a precursor of glutathione (GSH), an important enzyme of the cellular antioxidant system that is able to stimulate and sustain its intracellular levels, which detoxify ROS.

Conclusion

Doxorubicin induced cardiomyopathy, once developed, carries a poor prognosis and is frequently fatal. The present study showed that N-acetyl cysteine had beneficial effects that could ameliorate the severity of cardiac injury induced by Doxorubicin.

Extensive investigations are recommended to confirm and fully clarify the mechanism of N-acetyl cysteine effects on preventing Doxorubicin induced cardiomyopathy

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