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EXPERIMENTAL STUDY

The effects of N-acetylcysteine on cisplatin induced cardiotoxicity

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ABSTRACT
OBJECTIVES: Recent studies reported that oxidative stress is an important mechanism that contributes to cisplatin induced cardiotoxicity. In the present study, the effects of N-acetylcysteine (NAC), which is an antioxidant, on cisplatin induced cardiotoxicity were investigated in a rat model.
METHODS: Thirty two rats were separated into 4 equal groups: Control, NAC-250, CP (cisplatin), CP+NAC. Rats in the experimental groups were treated with a single dose of cisplatin intraperitoneally (ip) (10 mg/kg) and NAC (ip, 250 mg/kg) for 3 consecutive days. At the end of the experiment, cardiotoxicity was determined from plasma CK-MB, LDH, cTnI and cardiac myosin light chain-1 (CMLC-1) levels. In the tissue samples, total oxidant capacity (TOC), total antioxidant capacity (TAC), lipid hydroperoxide (ROOH) and thiol levels were measured. The hearts were also analyzed histopathologically.
RESULTS: It was determined that cisplatin increased the tissue TOC, ROOH levels and decreased TAC and thiol levels. NAC administration after cisplatin treatment was observed to have ameliorated histological and functional changes in heart.
CONCLUSIONS: In conclusion, the results of this experimental study suggested that oxidative stress had a serious effect on cisplatin cardiotoxicity, and NAC could be used as a therapeutic agent in addition to standard cisplatin treatment protocols *(Tab. 3, Fig. 1, Ref. 35)*. Text in PDF *www.elis.sk.*
KEY WORDS**:** rats, cisplatin, oxidative stress, cardiotoxicity, N-acetylcysteine.

Introduction

Chemotherapeutic agents, radiation therapy, and molecular targeted therapies used in oncology patients may cause damage to cardiovascular system through cardiac function and peripheral deteriorations and by increasing the often latently present changes in hemodynamic flow or worsening thrombotic events (1).

It has been shown that cisplatin, one of the most potent chemotherapeutic agents, is also associated with cardiotoxicity (2). Cisplatin is a standard component of treatment regimens for head and neck cancers, ovarian and cervical cancer, testicular cancer, non-small cell lung cancer, and bladder cancer. The mechanism of action has been linked to crosslinking with purine bases on DNA, which can lead to DNA damage, thereby inducing apoptosis in cancer cells (3).

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Cardiotoxicity induced by cisplatin includes echocardiography abnormalities, hypertension/ hypotension, angina and acute myocardial infarction, myocarditis, arrhythmias, cardiomyopathy, and congestive heart failure, and has been reported to be one of the major factors limiting a clinical usage of the drug (4, 5). Although the mechanisms underlying the cardiotoxic effects of cisplatin have not been fully identified, it has been shown that cardiotoxicity can result either from a direct toxic action of cisplatin on cardiac myocytes or from reactive oxygen species (ROS) production, followed by the induction of oxidative stress and the switch to a prothrombotic condition (4, 5).

In addition to the acute effects, delayed cardiovascular toxicity of cisplatin and cisplatin detected in the blood even 20 years after treatment makes the management of cardiotoxicity important (6). Although some cardioprotective strategies are available, the appropriateness of cardiovascular care remains suboptimal in clinical practice (7).

Increasing evidence showed that the use of antioxidants could be effective in ameliorating toxicity induced by cisplatin (8–11). Therefore, this study was planned to demonstrate the protective role of N-acetylcysteine (NAC), which has been shown to have antioxidant potential, against cisplatin-induced cardiotoxicity.

NAC, a glutathione precursor, can be given to patients at relatively high levels, has an excellent toxicity profile and is currently used in clinical practice (12). The mechanisms responsible for the beneficial effects of NAC have been associated not only to the antioxidant properties, but also to the anti-inflammatory effects (13).

NAC has also ben reported to show synergistic anti-tumor and anti-metastatic effects in murine models (14, 15).

The aim of this study was to evaluate the effects of cisplatin treatment on cardiological markers, especially cardiac myosin light chain-1 (CMLC-1) and oxidative stress, and the effect of NAC on cardiotoxicity

Methods

Approval for the study was granted by Erciyes University Local Ethics Committee for Animal Experiments (Ethic Committee No: 14/168). All the procedures were conducted in Erciyes University Experimental Research and Application Center Laboratory.

Animals

A total of 32 male Wistar albino rats, aged 24 weeks, each weighing 350–400 g, were housed in standard environmental conditions, with controlled temperature (22 ± 3 °C) and a 12-hours light/dark cycle. Free access was provided to food and water. All the procedures were performed in compliance with the Helsinki Declaration and International Guiding Principles for Biomedical Research Involving Animals.

Experimental design

Following a 1-week acclimatisation period, the rats were randomly separated into 4 groups of 8: control, cisplatin (CP), NAC (NAC-250) and cisplatin+NAC (CP+NAC). The control group was injected with saline (1 mL/kg). The CP group was treated with a single, intraperitoneal (ip) dose of cisplatin (10 mg/kg; Cisplatin DBL, 50 mg/100 mL, England). The NAC group was administered NAC ip (250 mg/kg; Asist, Bilim 300 mg/3 mL, 10 %, Istanbul, Turkey), and the CP+NAC group was treated with a single dose of cisplatin (10 mg/kg, ip) followed by NAC (250 mg/kg, ip) for 3 consecutive days. In this last combined treatment group, the NAC was administered 4 hours after the cisplatin injection to avoid interaction between the cisplatin and NAC (16).

At the end of the experiment, the rats were anaesthetized with an intraperitoneal injection of 80 mg/kg ketamine (Ketalar flacon, Pfizer; New York, USA) and 10 mg/kg xylazine (Rompun, Bayer; Leverkusen, Germany). After dissection, blood samples were taken from the abdominal aorta and the hearts were rapidly excised and trimmed of connecting tissue. Each heart was divided longitudinally into two parts, one of which was washed free of blood with ice-cold 0.9 % NaCl solution and then blotted dry and stored at –40 °C until used in the biochemical analyses. The other half of the heart was immediately fixed in 10% neutral formaldehyde for histological studies.

Biochemical analysis

Plasma CK-MB and LDH assays were performed photometrically on a Roche/Hitachi cobas c 701 system (Roche Diagnostics, Indianapolis, IN).

CMLC-1 and cardiac troponin I (cTnI) levels were measured using the enzyme-linked immunosorbent assay (ELISA) kit (Mybiosource rat CMLC-1 ELISA kit MBS266939; Cusabio rat cTnI ELISA kit, CSB-E08594r, respectively).

For tissue analysis, 0.2 mg of whole heart tissue was weighed for each heart. Samples were homogenized in ice with 2-mL phosphate buffer (consisting of 0.02 M EDTA) for thiol analysis and 0.015 M (pH 7.5) phosphate buffer for total oxidant capacity (TOC), total antioxidant capacity (TAC) and lipid hydroperoxide (ROOH) analysis. The samples were then centrifuged at 4 °C, 20.000 g, for 15 minutes. The supernatant was used for various biochemical determinations. Tissue ROOH, TAC, TOC and thiol results were obtained by proportioning to the protein values calculated by the Lowry method (17) in the same tissues.

ROOH levels were determined spectrophotometrically according to the modified ferrous oxidation with xylenol orange (FOX-2) method (18).

TOC and TAC levels were measured using commercial kits (Rel Assay, RL0024 and Rel Assay RL0017, respectively) in heart tissues.

Tissue thiol levels were measured spectrophotometrically according to the Ellman's assay using 5,5′-dithiobis, 2-nitrobenzoic acid (19).

Histological evaluation

For the evaluation under a light microscopy, the samples of heart tissue were fixed in 10 % buffered formalin for 48 hours then embedded in paraffin blocks after routine processing. For general morphological evaluation, sections approximately 5-µm in thickness were stained with hematoxylin and eosin (H & E). Examination was made of 5 sections for each slide and images were taken with a digital camera (Olympus C-5060, Tokyo, Japan) attached to a photomicroscope (Olympus BX51, Tokyo, Japan).

A histologist blinded to the groups performed the microscopic scoring of the heart sections. A score was given to represent the approximate extent of hemorrhage, vacuolization, and interstitial

Tab. 1. Effect of NAC on cisplatin induced changes in the biochemical indicators of cardiotoxicity.

Parameters	Control	CP	NAC-250	CP+NAC
CK-MB (U/L)	154 (101–211)	412 (392–582)*	109 (103–118)[a]	212 (198–223)[a,b]
LDH (U/L)	157 (145–260)	751 (578–794)*	119.5 (103–158)*[,a]	445.5 (422–473)*[,a,b]
CMLC-1 (ng/mL)	0.76 (0.46–0.88)	15.45 (13.34–23.22)*	0.77(0.67–1.02)[a]	5.82 (4.95–6.32)*[,a,b]
cTnI (pg/mL)	6.5 (0.75–11.75)	305.5 (298–350)*	13.5 (13–15.5)*[,a]	154.5(132.2–179.7)*[,a,b]

Data are expressed as median (min–max). n=8 for each experimental group. CP – cisplatin; NAC – N-acetylcysteine; LDH – lactate dehydrogenase; CK-MB – creatine kinase-MB; CMLC-1 – cardiac myosin light chain -1; cTnI cardiac troponin I. Significant findings were obtained, when compared to Control * ($p<0.05$), CP [a] ($p<0.05$) and NAC-250 [b] ($p<0.05$) groups.

Tab. 2. Effect of NAC on cisplatin induced changes in cardiac oxidative stress markers.

Parameters	Control (mean±SD)	CP (mean±SD)	NAC-250 (mean±SD)	CP+NAC (mean±SD)
TAC (mmol Trolox Eq /g protein)	0.741 ± 0.063	0.217 ± 0.098*	1.0 ± 0.140*, a	0.599 ± 0.056*, a, b
Thiol (nmol/g protein)	42.95± 3.57	32.60±3.92*	43.43± 4.23[a]	37.66 ±2.21* , a, b
Parameters	Control Median (min-max)	CP Median (min-max)	NAC-250 Median (min-max)	CP+NAC Median (min-max)
TOC (μmol H_2O_2 Eq/g protein)	1.82 (1.62–1.91)	3.31 (3.09– 3.56)*	1.83 (1.46–.2.04)[a]	2.19 (2.03–2.36)[a]
ROOH (nmol/g protein)	0.56 (0.41–0.74)	1.16 (1.0–1.31)*	0.62 (0.53–0.64)[a]	0.82 (0.76–0.89)*,a,b

Data are expressed as mean+SD or median (min–max). n = 8 for each experimental group. CP – cisplatin; NAC – N-acetylcysteine; TAC – Total Antioxidant Capacity; TOC – Total Oxidant Capacity; ROOH; Lipid Hydroperoxide. Significant findings were obtained, when compared to Control *($p<0.05$) and CP [a]($p<0.05$) groups and NAC-250 group [b]($p<0.05$).

Tab. 3. Semiquantitative analysis of histology of heart of rats treated with cisplatin with or without NAC.

Parameters	Control	CP	NAC-250	CP+NAC
Hemorrhage	0.00	2.5 (2.0–3.0)*	1.0 (0.0–2.0)*,a	2.0 (1.0–2.0)*,a,b
Interstitial edema	0.00	2.50 (2.0–3.0)*	1.0 (0.0–1.0)*,a	0.5 (0.0–1.0)*,a
Vacuolization	0.00	3.0 (2.0–3.0)*	0.5 (0.0–1.0)*,a	1.0 (1.0–1.0)*,a,b

Data are expressed as median (min–max). n = 8 for each experimental group. CP – cisplatin; NAC – N-acetylcysteine; Significant findings were obtained, when compared to Control *($p<0.05$) and CP [a]($p<0.05$) groups and NAC-250 group [b]($p<0.05$).

edema. These parameters were evaluated on a scale of 0-3, where 0= absent, 1 = mild, 2 = moderate, and 3=severe.

Statistical analysis

Statistical analysis of the study data was made with IBM SPSS Statistics version 23 software. Conformity of the variables to normal distribution was assessed with the Shapiro–Wilks test. The mean values were compared using the Student's t-test, One way ANOVA and the Kruskal–Wallis test. Data were considered statistically significant at a value of $p < .05$.

Results

The cardiac marker measurements are shown in Table 1. Cisplatin-induced cardiotoxicity was indicated by the significant increase in plasma CK-MB and LDH levels. NAC supplementation after cisplatin therapy reduced the increase in CK-MB observed in the cisplatin group.

Although still above the control levels, NAC administration after cisplatin decreased LDH levels compared to the cisplatin only group (Tab.1).

Plasma levels of CMLC-1 and cTnI in the cisplatin group were significantly higher than those of the control group. NAC supplementation after cisplatin significantly reduced CMLC-1 and cTnI levels compared to the cisplatin only group, but the levels remained above those of the control group (Tab. 1).

The TAC and TOC levels were significantly different between the CP group and the control group. NAC administration with cisplatin increased TAC levels, which were reduced in the cisplatin group, and were significantly lower than those of the control group. NAC supplementation significantly decreased high levels of TOC following cisplatin application compared to the control group (Tab. 2).

Increased ROOH levels in the CP group significantly decreased with NAC administration. Thiol levels decreased with cisplatin administration only, but the CP+NAC group showed a significant increase, while remaining significantly below the control group values (Tab. 2).

In the histopathological examination of heart tissues, the control group histopathology was normal with no evidence of hemorrhage, intertstitial edema or vacuolization (Fig. 1a). All the rats treated with cisplatin showed varying degrees of change in these parameters (Fig. 1b). In the CP+NAC group, interstitial edema and vacuolization of a mild degree was noted and was determined to be at a significantly lower level than seen in the cisplatin group (Fig. 1d). Hemorrhage was improved in the CP+NAC group compared to the cisplatin only group, but was still high. In the NAC-250 group, significantly high scores were obtained in all 3 parameters although not as prominent as in the cisplatin only group (Fig. 1c) (Tab. 3).

Discussion

Cisplatin is currently one of the most widely-used chemotherapeutic agents. However, there are serious side-effects in addition to its clinical efficacy (3). Although considered rare (20), cardiotoxicity as a diverse effect of cisplatin therapy has been reported in numerous studies (8, 21–24).

The development of cardiac disorders during or after the completion of cisplatin therapy is a serious problem and the main questions about this issue are how the development of cardiotoxicity can be predicted or monitored and what is the best prevention strategy (7). Since it has been reported that cisplatin induces oxidative damage in various tissues (2), it seems reasonable to investigate NAC supplementation as potential therapeutic approach for the attenuation of cisplatin toxicities.

The diagnostic approach to detect cardiac injury is echocardiography and the calculation of left ventricular ejection fraction. However, these diagnostic tests show a low sensitivity in the detection of cardiac damage especially in the early periods (25). Therefore, biochemical markers are preferred for the detection of cardiotoxicity in clinical and experimental studies.

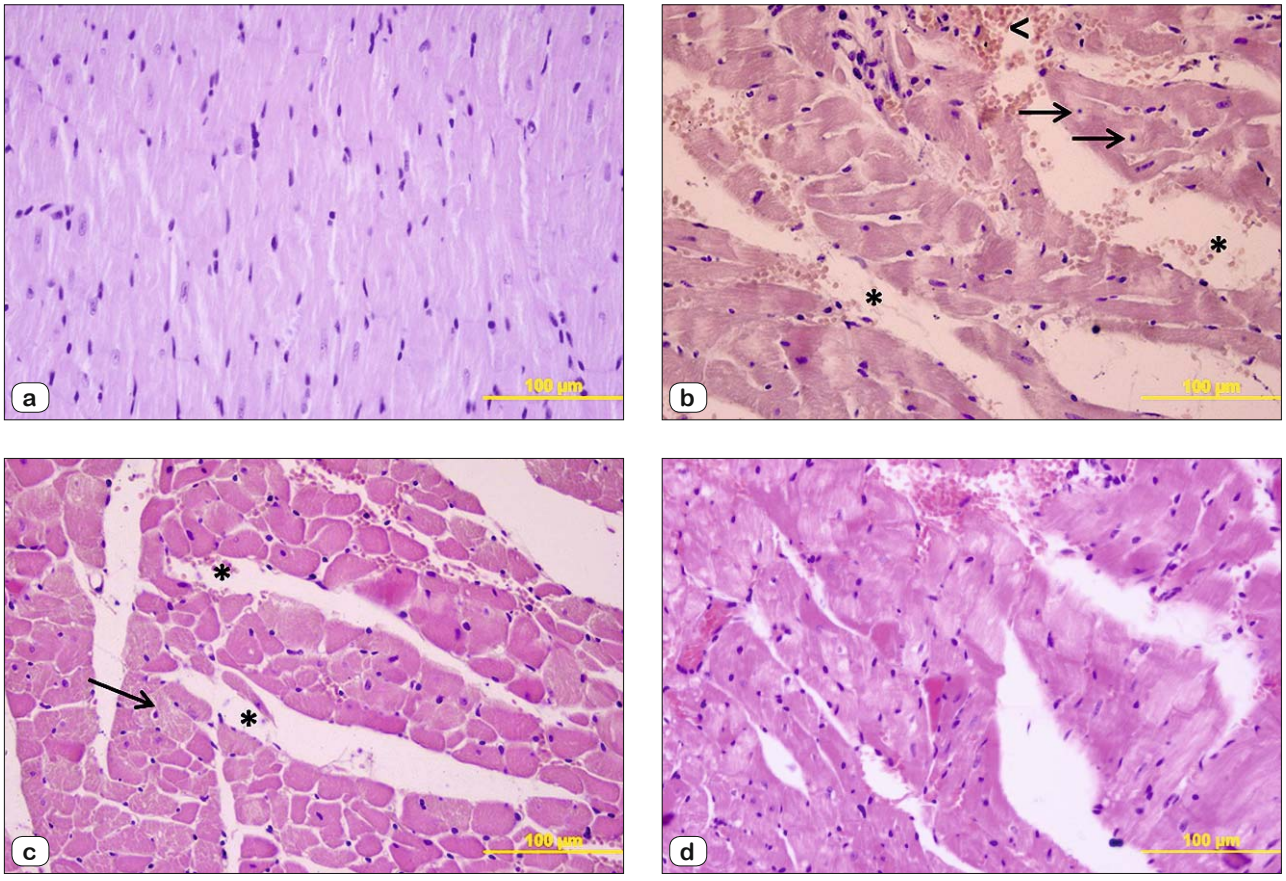

Fig. 1. The morphology of the heart tissues. The images were representatives the H&E-stained sections of heart from experimental groups (a) Cardiac tissue showing the normal structure in the control group (b) Cisplatin-injured cardiac tissue obtained from cisplatin group (c) Only NAC treated group (d) The cardiac tissue obtained from the cisplatin plus NAC treated group (Original magnification=40x, Scale bar=100 µm) Arrow (→): hemorrhage; arrow head (<): interstitial edema; Star (*): vacuolization.

In the present study, CK-MB and LDH levels significantly increased in addition to cTnI and CMLC-1 plasma levels in cisplatin-treated animals. The elevation of these enzymes in serum/plasma are known to be diagnostic markers for cardiac injury (26).

To the best of our knowledge, this is the first study to have reported changes in CMLC-1 in cisplatin cardiotoxicity. It is thought that CMLC-1 has the physiological function of modulating interaction between myosin and actin. Following myocardial injury, at 3 ± 6 hours after the onset of pain, a release of the unbound cystolic fraction allows CMLC-1 should be detected in the circulation. After approximately 4 days, peak values will be recorded and elevated plasma concentrations can continue for 10 ± 14 days, which reflects the continued release from infarcted myofilaments (27).

Although LDH and especially CK-MB are still often used, cTnI is recommended for the determination of myocardial injuries because of its sensitivity and specificity (28). The results of the current study demonstrated that the change of CMLC-1 levels was similar to cTnI highly sensitive and specific markers of myocardial injury in terms of cisplatin cardiotoxicity.

Previous studies reported similar elevations in CK-MB, LDH (23, 29) and cTnI levels (21, 23) after cisplatin administration.

Increased lipid peroxidation with cisplatin administration could be the reason for increased cardiac markers in rat plasma. As cisplatin creates different types of bi-functional adducts with cellular DNA, it is generally regarded as an anti-cancer drug that has destructive effects on the DNA. Recent data have also suggested that in addition to DNA damage, cisplatin induces ROS, thereby triggering cell death resulting in irreversible changes in the myocardial membrane structures, functions and integrity with subsequent leakage of cardiac enzymes (30). Phospholipids located in the mitochondrial and cell membrane, cardiolipin, phosphatidylcholine, and phosphatidylethanolamine are composed of polyunsaturated fatty acids, which are especially susceptible to peroxidation by ROS (31). Likewise, El-Sawalhi et al (10) reported that cisplatin administration (ip, 7 mg/kg) resulted in the elevation of CK-MB through irreversible modifications and damage to the myocyte membranes that emerge with lipid peroxidation.

It has also been reported that NADPH oxidase, which is one of the major sources of ROS, increases the ROS production through cisplatin (10) and some antioxidant enzymes, such as: superoxide dismutase and catalase, have been reduced with cisplatin treatment (22, 24, 29).

In the current study, an increased oxidative stress was demonstrated by the increased TOS and decreased TAS levels, and lipid peroxidation was confirmed with the ROOH measurement. However, to date there has been no other study showing the change of lipid hydroperoxide levels in cisplatin-induced cardiotoxicity, and lipid peroxidation has been evaluated by increased malondialdehyde (23, 29) and thiobarbituric acid reactive substances (22) levels in the previous studies.

In addition to ROS production, cisplatin contributes to the emergence of oxidative stress by converting into a highly reactive form in cells, which can rapidly react with thiol-containing molecules, such as reduced glutathione (GSH) to form a conjugate and this might account for the GSH depletion (32). The reduction of GSH could also be explained by a decreased activity of glutathione reductase resulting from cisplatin attack (33). The intracellular redox homeostasis is maintained by the thiol group (-SH) containing molecules (2). In previous studies it has been indicated that total thiol (21) and GSH (22, 23, 29) levels decreased with cisplatin administration at several doses ranging from 5 to 10 mg/kg rat body weight. Likewise, in the present study, decreased thiol levels were reported with cisplatin (ip, 10 mg/kg) treatment.

Although there is no similar finding in literature, the results of the present study showed significantly increased cTnI levels and decreased LDH levels in the NAC-250 group. There was also a numerical decrease in CK-MB levels. In the same group (NAC-250), the damage observed histopathologically was consistent with increased cTnI levels. Decreased levels of LDH and CK-MB could not be explained. However, it has been previously reported that because CK-MB and LDH have higher molecular weights (84 kDa and 135 kDa respectively) than cTnI (24 kDa), CK-MB and LDH could only be released after an irreversible damage (34). This hypothesis could explain increased levels of cTnI in the NAC-250 group, which displayed a mild damage. However, according to this hypothesis, similar CMLC-1 results to cTnI levels should be obtained, which could be attributed to the late plasma peak of CMLC-1 compared to cTnI (27). It would not be correct to speculate and propose any explanation for this cardiotoxic effect of NAC, so this question remains open for further investigations. However, a similar change in oxidative stress parameters was not seen with NAC administration.

Recent studies demonstrated that cisplatin treatment disrupts redox homeostasis by production of ROS and reduction of antioxidative enzyme activity and the use of antioxidants, especially those including thiol groups, such as NAC, has been suggested (35). However, most of these findings have been the results of experimental and clinical investigations on kidneys, as cisplatin shows evident nephrotoxicity, and the lack of data, related to the effects of cisplatin on cardiac muscles, remains (9).

The present study demonstrated that elevated levels of cardiac injury markers (CK-MB, LDH, cTnI and CMLC-1) were significantly reduced by NAC, but only the CK-MB values were statistically similar to those of the control group. It was reported that administration of NAC (ip 500 mg/kg, for 5 weeks) during cisplatin treatment (ip 5 mg/kg, for 5 weeks) reduced LDH and CK levels compared to the group treated with cisplatin alone (8). This could be attributed to the preventive action of NAC on membrane phospholipids.

The current study also showed that NAC significantly reduced TOC and ROOH levels while increasing TAC and thiol levels, compared to the control group. These results are in line with those of Rosic et al (8), who reported that NAC treatment significantly increased GSH levels in cisplatin-treated rat hearts.

However, in the current study NAC administration alone did no alter thiol levels in rat heart tissues.

Rosic et al (8) documented similar results and attributed this outcome to the fact that NAC applied at a dose of 500 mg/kg (ip) for 5 weeks, did not act as a glutathione precursor, but as a direct antioxidant.

Finally, the biochemical findings obtained from this study were confirmed by the histopathological examination of heart tissues, which showed severe focal hemorrhage, interstitial oedema and vacuolization. These histopathological observations supported the view that NAC may protect myocardial tissue from cisplatin-induced cardiotoxicity as demonstrated by amelioration of the myocardial injury.

As most of the cardiac injury markers in the current study and the TOC and ROOH levels were still above those of the control group, it can be said that NAC at these doses and durations was not fully successful. As NAC has been found to undergo extensive metabolism on first passage through the liver, the administration of NAC can be recommended for longer periods, and/or at higher doses or intravenously.

Conclusion

The results of this study showed that biochemically and histopathologically, cisplatin increased CK-MB, LDH, cTnI, CMCL-1, ROOH and TOC levels, and decreased thiol and TAC levels in the cardiac tissue, thereby leading to oxidative damage. Although, NAC at doses of 250 mg/kg rat body weight could not completely prevent the cardiac injury resulting from cisplatin-induced oxidative cardiac toxicity, the extent of the cardiac damage was diminished compared to the cisplatin treatment alone.

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