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Cardiac Troponin-I (cTnI) a Biomarker of Cardiac Injuries Induced by Doxorubicin Alone and in Combination with Ciprofloxacin, Following Acute and Chronic Dose Protocol in Sprague Dawley Rats

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Abstract: The present study investigates the release of cardiac troponin-I (cTnI) as a biomarker of cardiac injuries induced by doxorubicin (Doxo) alone and along with ciprofloxacin (Cipro), following acute and chronic dose protocol in Sprague Dawley rats. In chronic protocol, rats were given multiple intra-peritoneal (i.p) injections of Doxo (1 or 2.5 mg kg⁻¹) alone or in combination with Cipro (20 mg kg⁻¹ daily) and a placebo control. Whereas in acute protocol, rats were subjected to receive single i.p. injection of Doxo (6 or 15 mg kg⁻¹) alone or along with Cipro (20 mg kg⁻¹) and placebo treatment with saline (control). The plasma levels of cTnI were measured by using Enzyme-linked Immuno Sorbent Assay (ELISA) technique. All the treated groups, following acute or chronic dose protocol showed significant increase in cTnI plasma level from 137-248% in comparison to control (p<0.0001). The cTnI plasma levels increased in dose dependent manner after following acute and chronic dose protocol. The difference between two doses following chronic (1 mg kg⁻¹ vs. 2.5 mg kg⁻¹) and acute (6 vs. 15 mg kg⁻¹) administration was 34.6 and 31.5%, respectively. Results of this investigation suggest that Doxo alone and in combination with Cipro from both the chronic and acute groups showed cardio-toxicity (release of cTnI). To our knowledge this is the first description towards Doxo-Cipro is induced cardiotoxicity and could be a bridge between preclinical and clinical practice for physicians in making an expert opinion dealing with above mentioned group.

Key words: Cardiotoxicity, ciprofloxacin, cardiac troponin-I (cTnI), doxorubicin and drug-drug interaction

INTRODUCTION

Exposure to drugs, used to treat cancer and certain types of infections, leads to an inadvertent and undesirable cardiac toxicity (Falagas *et al.*, 2007; Saracoglu *et al.*, 2009). Besides, patients receiving chemotherapy usually suffer from certain other diseases like diabetes, cardiovascular disorders and certain kinds of infections. During the course of chemotherapy patient's body becomes more prone to different kinds of infections, due to low white blood cell counts (neutropenia) (Gaftar-Gvili *et al.*, 2005). The prevalence of poly-pharmacy in chemotherapy may lead to drug-drug interactions.

Doxorubicin (Doxo), a chemotherapeutic agent, is potentially used in clinical practice to treat solid and hematological malignancies but cardiotoxicity compromises its clinical utility (Herman *et al.*, 1998). Cardiotoxicity covers a spectrum of disorders from mild transient blood pressure to changes in electrocardiographic which may lead to arrhythmias and myocardial ischemia/infarction that may end in left

ventricular dysfunction or congestive heart failure (Shakir and Rasul, 2009). These disorders may occur during or soon after chemotherapy sometimes within days or after weeks of treatment, or may not be noticeable until months and sometimes years. Therefore, the present study was divided into two major groups acute and chronic, to study the dose behavior on cardiac functions of Sprague Dawley rats.

Ciprofloxacin (Cipro) is well tolerated and effective against wide variety of pathogenic bacteria including gram-positive and negative organisms (Shahzadi *et al.*, 2011). It occupies distinct place in chemotherapy for immuno-compromised patients. It augments the action of doxorubicin and epirubicin, used to prevent bladder, colorectal and prostate cancer in humans (Aranha *et al.*, 2003; Gurtowska *et al.*, 2010; Herold *et al.*, 2002). Recent studies indicated Cipro induced hepatic and cardiac toxicities (Adikwu and Brambaifa, 2012). Investigation revealed that Cipro induces cardiotoxicity by increasing the QT and QTC interval, action potential prolongation both in animal and as well as in clinical practice (Prabhakar and Krahn, 2004).

Cardiac cell death can be predictable by histological findings but it cannot be frequently used during pharmacology investigations as it is relatively time-consuming and expensive technique as compared to measurement of a serum or plasma-based biomarker troponin. Increased levels of cardiac troponins-I (cTnI) and troponins-T (cTnT) in the blood stream have become a well-established biomarker with high sensitivity and specificity for myocardial infraction or necrosis in man and animals (Antman, 2002; O'Brien *et al.*, 2006; Sarko and Pollack Jr., 2002). Troponin-I is a heteromere protein in the troponin complex of cardiomyocytes. Specific N-terminal sequence of cardiac cTnI distinguishes it from skeletal muscle isoforms of TnI (Bertazzoli *et al.*, 2014). Release of cTnI from myocardium is proportional to the size and extent of tissue injury in several animal models of cardiotoxicity (O'Brien *et al.*, 2006).

Our previous study regarding the Cipro and Doxo combination showed a significant pharmacokinetic interaction. The addition of Cipro along with Doxo increased the plasma concentration of Doxo of Sprague Dawley rats (Shahzadi and Yazici, 2014). So, it is hypothesized that increase in plasma Doxo levels after Cipro use can lead to increase myocardial toxicity. Therefore, the present study was designed to investigate the myocardial damage induced by low and high doses of Doxo given in acute and chronic schedule alone and in combination with Cipro by measuring cTnI levels in rat plasma.

MATERIALS AND METHODS

Experimental Animals Ethics Committee of Istanbul University, Cerrahpasa Faculty of Medicine approved the following study. About 80 healthy adult male Sprague Dawley rats, weighing 250-300 g at the start of experiment were used to conduct this study.

Drugs: Locally purchased formulation of Cipro and Doxo were used, Ciproxin Flacon (400 mg; Bayer, Turkey) and Adriamycin Ampoule (10 mg/5 mL; Deva Pharmaceuticals, Turkey), respectively.

Dose schedules: Rats were randomly divided into two main groups i.e., acute and chronic and 10 subgroups, having 8 animals in each to receive the same cumulative doses of Doxo but in different schedules. The doses and schedules are given as follows.

Rats were given different doses (6 and 15 mg kg⁻¹) of Doxo alone and in combination with Cipro (20 mg kg⁻¹; daily) as single intraperitoneal injection (i.p.).

Multiple i.p. injections of Doxo (1 and 2.5 mg kg⁻¹ twice a week, cumulative dose: 6 and 15 mg kg⁻¹, respectively) alone and along with Cipro (20 mg kg⁻¹, i.p. daily) were given to rats for the duration of three weeks. Whereas, control group rats received serum physiological solution.

Plasma analysis: Blood samples were collected on the third day of last injection following acute and chronic treatment protocol. Plasma cTnI levels were determined using a commercially available Enzyme-Linked Immuno Sorbent Assays (ELISA) kits (East Biopharm, China).

General toxicity: General toxicity was estimated by measuring the survival percentage and by considering the relative organ weight. At the end of chronic and acute dose protocols, rats were sacrificed by cervical dislocation; heart, liver, testes, spleen and kidneys were removed quickly after visual inspection and their weights were recorded. Organ weight was expressed as relative organ weight, i.e., as follows:

$$\text{Weight of an organ relative to the body weight of the rat} = \frac{\text{g organ weight}}{\text{g body weight}} \times 100$$

Statistical analysis: All values are presented as Mean±SE. Statistical significance (p<0.05) was determined by one-way ANOVA followed by *post hoc* Tukeys test (Graph Pad Prism 4.0). Whereas, two-way ANOVA was carried out to study the interaction between Doxo and Cipro groups.

RESULTS

General toxicity

Survival percentage: Rats treated with chronic high dose of Doxo alone (2.5 mg kg⁻¹) showed 50% mortality rate, which was significantly higher than that of control, Doxo (1 mg kg⁻¹) and Doxo+Cipro (1 +20 mg kg⁻¹) treated rats (p<0.05, Fig. 1). Whereas, mortality rate was 38% in rats treated with Doxo+Cipro (2.5 +20 mg kg⁻¹) in comparison to control. No mortality was observed in rats of chronic Doxo (1 mg kg⁻¹) and Doxo+Cipro (1+20 mg kg⁻¹). The rats of acute group under low and high doses (6 and 15 mg kg⁻¹ or with Cipro 20 mg kg⁻¹) therapy maintained 100% survival.

Relative organ weight: The relative heart weight of rats treated with Doxo 2.5 mg kg⁻¹ showed significant reduction than that of control (34.6%). Whereas, 56% of reduction was restored by the addition of Cipro (20 mg kg⁻¹) to this group (p<0.0001). There was no significant difference between the other groups (Fig. 2).

The relative liver weight of rats treated with Doxo 1 mg kg⁻¹ showed significant increase of 34.6% with respect to control (p<0.01). However, Doxo+Cipro (2.5+20 mg kg⁻¹) in comparison to Doxo 1 mg kg⁻¹ showed a significantly minor liver atrophy (22%, Fig. 3).

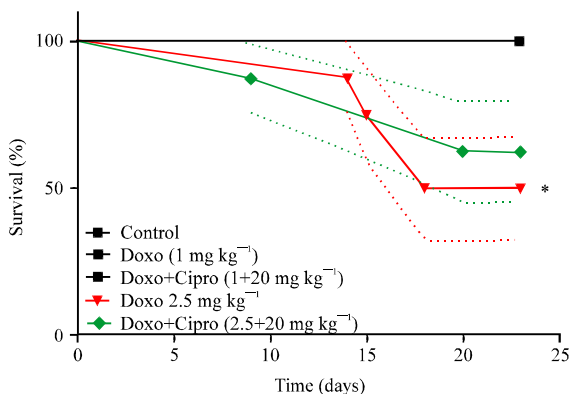


Fig. 1: Percentage survival (Kaplan-Meier Plot) of rats treated with multiple i.p. injection of saline, Doxo (1 or 2.5 mg kg⁻¹) alone and in combination with Cipro (20 mg kg⁻¹ daily) for the duration of 3 weeks and till the end of experiment. Dotted lines representing SE. *p<0.05 vs. Control, Doxo (2.5 mg kg⁻¹), Doxo+Cipro (2.5+20 mg kg⁻¹)

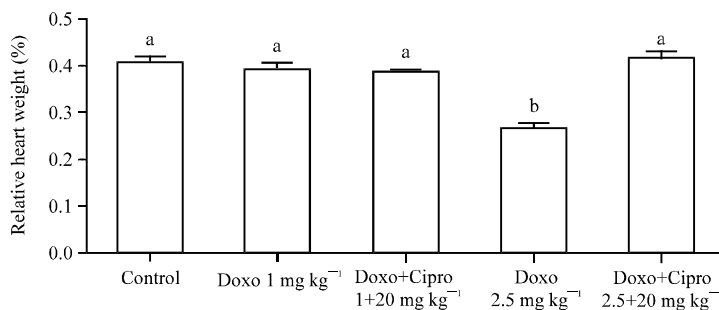


Fig. 2: Effect of Doxo alone and along with Cipro on relative heart weight of Sprague Dawley rats. Values are the Means±S.E, bars having similar letters do not differ significantly (p<0.0001)

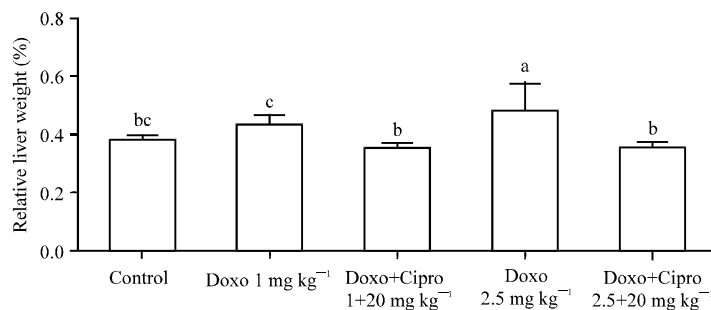


Fig. 3: Effect of Doxo alone and along with Cipro on relative liver weight of Sprague Dawley rats. Values are the Means±S.E, bars having similar letters do not differ significantly (p<0.01)

All the treated group except Doxo+Cipro (1+20 mg kg⁻¹) showed a significant reduction of 76-80% in relative spleen weight than that of control (p<0.0001, Fig. 4).

A significant dose dependent increase (26%) in relative kidney weight was observed in Doxo (2.5 mg kg⁻¹) group (p<0.0001). While, it was restored to control values when Cipro (20 mg kg⁻¹) was added to this group (Fig. 5).

Relative testes weight of rats treated with Doxo (2.5 mg kg⁻¹) showed a significant reduction of (50%) (p<0.0001). However, the addition of Cipro (20 mg kg⁻¹) to this group brought the levels back to normal (Fig. 6).

Whereas, rats under the acute schedule of Doxo or Doxo+Cipro groups did not show significant variations in organ weights.

Cardiac troponin-I (cTnI) plasma concentration: Extend of cardiac injury induced by Doxo-Cipro following acute and chronic dose protocols was determined by estimating the plasma cTnI levels of treated rats. The rats given saline solution showed 1.317±0.12 ng mL⁻¹ cTnI levels during the experiment. The administration of Doxo (1 or 2.5 mg kg⁻¹) alone and along with Cipro (20 mg kg⁻¹) showed a significant increase (p<0.0001) in cTnI levels in comparison to control (Fig. 7). The highest concentration

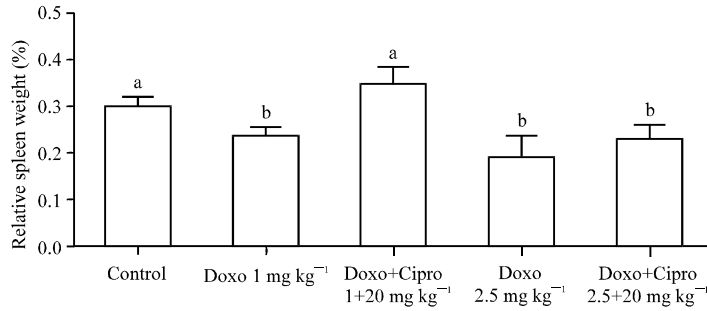


Fig. 4: Effect of Doxo alone and along with Cipro on relative spleen weight of Sprague Dawley rats. Values are the Means±S.E, bars having similar letters do not differ significantly ($p < 0.0001$)

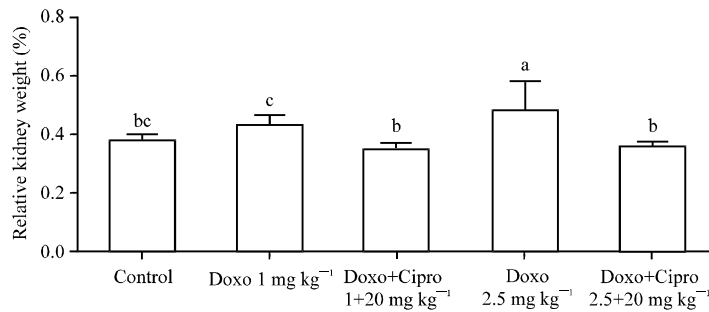


Fig. 5: Effect of Doxo alone and along with Cipro on relative kidney weight of Sprague Dawley rats. Values are the Means±S.E, bars having similar letters do not differ significantly ($p < 0.0001$)

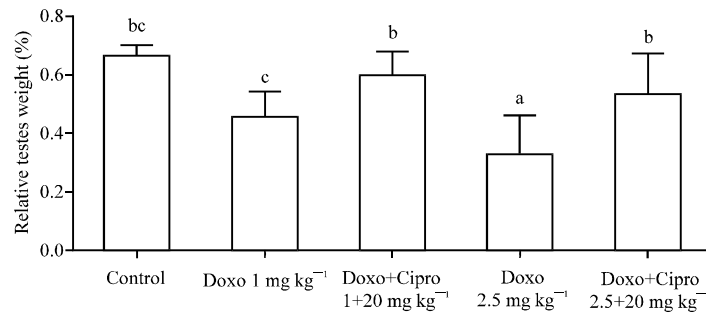


Fig. 6: Effect of Doxo alone and along with Cipro on relative testes weight of Sprague Dawley rats. Values are the Means±S.E, bars having similar letters do not differ significantly ($p < 0.0001$)

of cTnI was found in rats treated with high dose of Doxo (2.5 mg kg^{-1}) alone followed by Doxo+Cipro ($2.5+20 \text{ mg kg}^{-1}$) (4.405 ± 0.07464 , $4.335 \pm 0.1913 \text{ ng mL}^{-1}$, respectively).

Similar trend was observed in the plasma cTnI concentration of rats that followed acute protocol. Single i.p. injection of low or high doses of Doxo (6 or 15 mg kg^{-1}) alone and in combination with Cipro (20 mg kg^{-1}) showed significant increase in cTnI plasma levels than that of control ($p < 0.0001$, Fig. 7). The highest increase in plasma cTnI concentration was observed in rats given high dose of Doxo (15 mg kg^{-1}) alone

($3.921 \pm 0.2018 \text{ ng mL}^{-1}$) closely followed by Doxo+Cipro ($15+20 \text{ mg kg}^{-1}$), ($3.925 \pm 0.2836 \text{ ng mL}^{-1}$).

The significance was at par, when plasma cTnI levels of rats treated with chronic low dose Doxo (1 mg kg^{-1} , cumulative 6 mg kg^{-1}) was compared with acute low dose Doxo (6 mg kg^{-1}) alone or along with Cipro (Fig. 8). Similarly, chronic high dose Doxo (2.5 mg kg^{-1} ; Cumulative 15 mg kg^{-1}) and acute high dose Doxo (15 mg kg^{-1}) groups, either alone or in combination with Cipro were found to be statistically similar in terms of cTnI plasma levels (Fig. 9).

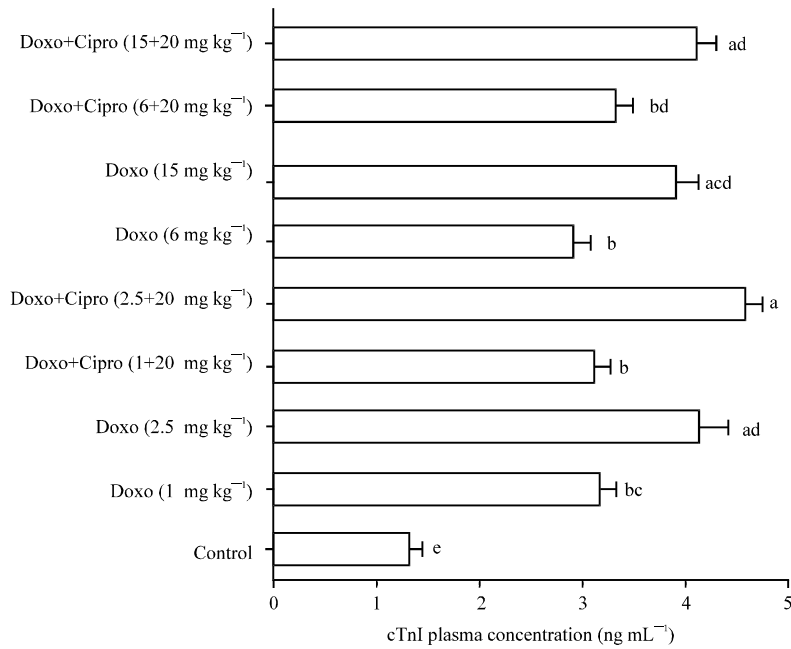


Fig. 7: cTnI plasma levels of Doxo alone and along with Cipro following chronic and acute dose protocols. Values are the Means±S.E, bars with similar letters do not differ significantly ($p < 0.0001$)

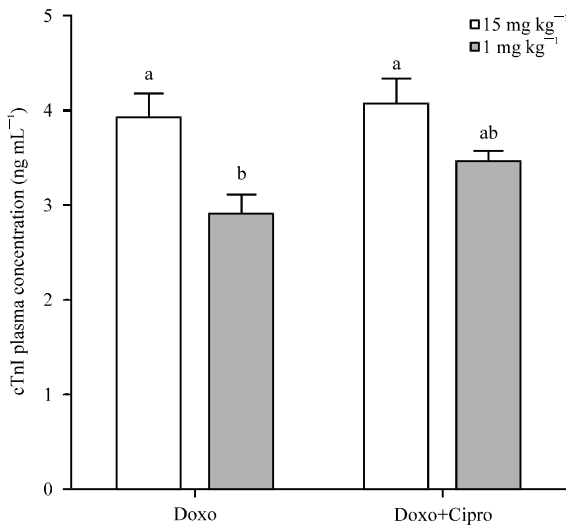


Fig. 8: Cardiac troponin-I (cTnI) plasma concentration of rats administered with acute Doxo alone and along with Cipro. Values are the Means±S.E, bars with similar letters do not differ significantly ($p < 0.05$)

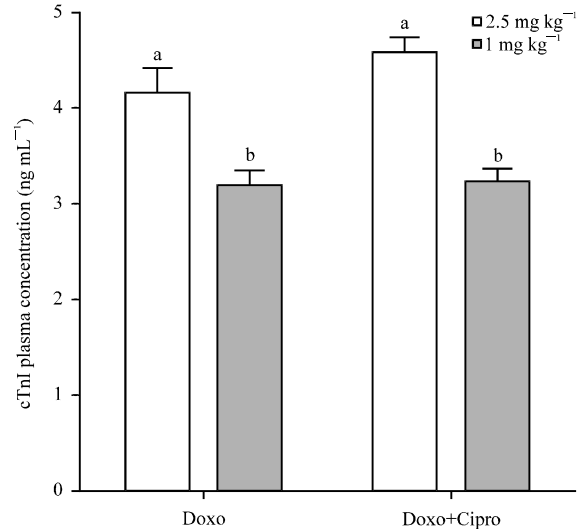


Fig. 9: Cardiac troponin-I (cTnI) plasma concentration of rats treated chronically with Doxo alone and along with Cipro. Values are the Means±S.E, bars with similar letters do not differ significantly ($p < 0.05$)

DISCUSSION

A dose dependent cumulative cardio-toxicity of Doxo always remains a major clinical concern and led to the development of several animal models of chronic and acute cardio-toxicity (Bertazzoli *et al.*, 2014; Herman *et al.*,

2000). Cipro is a well-known broad spectrum antibiotic that has been successfully used in daily clinical practice as well as in oncology as a supportive therapy for immuno-compromised patients. So, the hypothesis behind this experiment was to study the interaction of Cipro with

Doxo and to assess its cardio-protective or cardio-toxic effects, following different dose protocols in Sprague Dawley rats.

The use of plasma levels of cardiac troponin-I (cTnI) has been well-established in clinical practice to measure cardio-toxicity in cancer patients (Lipshultz *et al.*, 2004; Urbanova *et al.*, 2009). It has also been used as biomarker to identify the extent of cardiac injury in preclinical studies (Bertinchant *et al.*, 2003; Herman *et al.*, 1998). The current report showed an increase in cTnI levels at each dose of Doxo alone or in combination with Cipro followed either acute or chronic dose schedule. However, the maximum cTnI concentration was observed in rats treated with high doses of Doxo alone (2.5 and 15 mg kg⁻¹ in chronic and acute dose schedules, respectively) and in combination with Cipro (2.5+20 mg kg⁻¹ and 15+20 mg kg⁻¹). The probable cause of increase in cTnI levels is damage to myocardium which could be due to anemic hypoxia resulting due to alteration in erythropoiesis (Wallace *et al.*, 2004). A dose dependent increase in cardiac lesions was also reported in Fischer rats treated with Doxo 1-2 mg kg⁻¹ upto 10-14 weeks (Mettler *et al.*, 1977) and spontaneously hypertensive Wister-Kyoto rats treated with cumulative dose of 12 mg kg⁻¹ (Herman *et al.*, 1985). Literature indicated that myocardial injury increases with increase in cumulative dose, as observed in Sprague Dawley rats treated with the cumulative dose of 10, 13.5 and 18 mg kg⁻¹ and in rabbits administered with 11, 14, 16 mg kg⁻¹ of Doxo (Solcia *et al.*, 1981) and same is confirmed by our study.

On the other hand, literature showed that Cipro causes cardiotoxicity by initiating the oxidative stress in the heart, nitric oxide is thought to play a key role in this toxicity in juvenile rats (Saracoglu *et al.*, 2009). Alternative hypotheses can be the involvement of prostaglandins (Das, 1981), histamine (Bristow *et al.*, 1983) and C-13 anthracycline metabolites (Olson *et al.*, 1988) as mediators of cardiac toxicity. However, in the current study Cipro did not cause any significant change in plasma cTnI levels, but our previous reports indicated a significant increase in Doxo plasma concentrations after the concomitant use (Shahzadi and Yazici, 2014). This shows that there is a pharmacokinetic interaction but this interaction did not cause any significant effects on plasma cTnI concentration. It can be speculated that the non-significant increase in cTnI plasma levels of Cipro treated group in comparison to Doxo group can be due to the use of minimum effective dose of Cipro. The minimum effective dose was selected to reduce the other possible toxicities induced by Cipro. However, further investigation is needed to evaluate the exact role of Cipro in myocardial injury.

Doxo is administered systemically so it may harm the healthy tissues as well, leading to unfavorable effects to tissues, which ultimately, reduces the quality of life in cancer patients. In this study, Doxo alone and in combination with Cipro following different dose schedule in Sprague Dawley rats was tested to examine their effects on different body organs. Results indicated that the rats treated with high dose of Doxo (2.5 mg kg⁻¹) alone under chronic dose schedule leads to atrophy of rats heart (Fig. 2), that can be attributed to apoptosis and necrosis of heart tissues which is usually observed when cardiac cells are exposed to Doxo (Zhu *et al.*, 2009). The loss of cardiac structure and deteriorated function usually observed in long-term treatment with Doxo (Migrino *et al.*, 2008; Shakir and Rasul, 2009) which is in accordance with the findings of the current study recorded under chronic model. Whereas, the results of the following study also showed that Cipro helps in the restoration of heart cells by decreasing the Doxo induced necrosis.

Relative liver weight showed non-significant differences in comparison to control while interestingly a slight increase in liver weight was observed in Doxo 1 mg kg⁻¹ treated rats in comparison to Doxo+Cipro (2.5+20 mg kg⁻¹) (Fig. 3). The slight increase in liver weight at low dose Doxo is not clear, however, the possibility of hyperlipidemia cannot be ruled out.

All the treatment groups showed decrease in relative spleen weight except Doxo+Cipro (1+20 mg kg⁻¹) when compared with control. Drastic decrease in relative spleen weight was observed in the rats treated with high dose of Doxo alone (2.5 mg kg⁻¹ following chronic schedule) which can be correlated with changes in hematological parameters (Herman *et al.*, 1985). Hematological changes such as low RBC, Hb and HCT counts was observed in patients suffering from breast cancer (Panis *et al.*, 2012) as well seen in hypertensive and Wister-Kyoto rats treated with 12 mg kg⁻¹ cumulative dose of Doxo (Herman *et al.*, 1985). Literature also showed bone marrow depression in patient undergoing chemotherapy with Doxo (Carvalho *et al.*, 2009; Vici *et al.*, 2011).

Relative weight of kidney was increased in rats treated with Doxo which is in accordance to earlier findings of Sharkey *et al.* (2013), who reported that this increase was the result of glomerular damage, as observed in patients undergoing Doxo treatment (Bardi *et al.*, 2007). In present study, application of Doxo along with Cipro at all doses improved the kidney weight of rats; literature also confirmed that Cipro does not exert nephrotoxicity in rats (Baykal *et al.*, 2005).

Testicular atrophy was observed in rats treated with chronic Doxo alone both with low and high doses (1 and 2.5 mg kg⁻¹). The effect was severe at chronic high dose

of Doxo. These findings are in accordance with previous findings which suggest that Doxo treated rats showed testicular toxicities (Xin *et al.*, 2012). It is evident from the literature that chemotherapeutic agents also affects the non-target tissues like testes (Da Cunha *et al.*, 1983; Suter *et al.*, 1997) and Doxo inhibits spermatogenesis (Howell and Shalet, 2005; Yeh *et al.*, 2007) and finally leads to infertility. The underlying mechanism for Doxo induced testicular toxicity is yet not clear but recent findings strongly suggest the oxidative stress from lipid peroxidation (Atessahin *et al.*, 2006; Yeh *et al.*, 2007) and cellular apoptosis (Shinoda *et al.*, 1999) as major reasons. The reason regarding the improvement in relative testes weight in rats treated with Cipro+Doxo in present study is not clear and needs further investigation. The increased toxic effects can be subjected to pharmacokinetic interaction. Our previous study showed an increase in plasma Doxo concentration when Doxo was combined with Cipro (Shahzadi and Yazici, 2014).

Whereas, interestingly organ weights showed non-significant differences in acute model which was contrasting to the previous findings (Minotti *et al.*, 2004; Pereira *et al.*, 2011; Weiss, 1992) who reported that acute model caused hypertrophy of heart. In addition to this, it is also reported that a single injection of 10 mg kg⁻¹ Doxo initiated decrease in heart mass followed by a restoration to control values (Lushnikova *et al.*, 2004) which was not observed in our acute model that can be due to low sample size or may be the use of different traits of rats. As there are genetic differences which can alter the metabolism and vulnerability to toxic agents (Kacew, 1996; Mas *et al.*, 2000). So, further investigation is required for the better interpretation of results in acute and chronic model of Doxo along with Cipro, because both of these drugs are commonly prescribed in chemotherapy and can lead to unseen toxicity.

CONCLUSION

Findings of this study confirm that Doxo showed dose dependent cardio-toxicity and Cipro did not show any significant change in cTnI levels. Whereas, in general Cipro played an important role in improvement of organs weight. This combination needs further investigation in terms of cardio-toxicity so that this combination can be safely prescribed in clinical practice to the patients undergoing chemotherapy with Doxo and need Cipro for being immune-compromised or to treat other complications.

Abberivations

Cipro	=	Ciprofloxacin
cTnI	=	Cardiac Troponin-I
Doxo	=	Doxorubicin
i.p	=	Intraperitoneal

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