Original Articles

CARDIOTOXIC EFFECTS OF SEQUENTIAL ADMINISTRATION OF DOXORUBICIN AND PACLITAXEL IN RATS

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يشكل معدل الحدوث العالي لتسمم القلب من تآلف الدوكسوروبوسين والباكليتاكسل بالمقارنة مع الدوكسوروبوسين منفرداً عقبةً تجاه العلاج الكيماوي الفعال. لذلك قمنا بدراسة تأثير تعاقب ووقت تعاطي كلا الدوائين على شدة تسمم القلب لهذا التآلف. لذلك تم تقسيم ذكور جرذان الويستر إلى سبع مجموعات، حيث أعطي الدوكسوروبوسين عن طريق البريتون بجرعة مفردة مقدارها 5 مغ/كغ كل يومين، وجرعتان كل أسبوع بإجمالي جرعة تراكمية مقدارها 20 مغ/كغ. أما بالباكليتاكسل فقد أعطي عن طريق البريتون بجرعة مقدارها 20 مغ/كغ كل يومين. وقد تم حقن الدوائين إما بصورة مفردة أو تعاقبياً كتآلف. ففي إحدى الحالات، سبق الدوكسوروبوسين الباكليتاكسل بثلاثين دقيقة أو 24 ساعة، وفي حالة أخرى، سبق الباكليتاكسل الدوكسوروبوسين بثلاثين دقيقة أو 24 ساعة. وتم تقييم تسمم القلب بالفحصين البيوكيميائي والهستوباثولوجي بعد 48 ساعة من آخر جرعة دوكسوروبوسين. وقد تجلى تسمم القلب المستحث بالدوكسوروبوسين بالتغيرات البيوكيميائية الشاذة بما في ذلك الزيادة الواضحة في نشاط كرياتين فوسفوكيناز أيزونزيم في المصل، واللاكتات ديهيدروجيناز، والجلوتاثيون بيروكسيداز، وأسبارتات أمينوترانسفيراز. كما أظهر نسيج عضلة القلب المأخوذ من الجرذان المعالجة بالدوكسوروبوسين زيادات معنوية في إنتاج المالونالدهيد في إجمالي مستويات النترات/ النتريت (NOx) توازي استنزاف "احتياطي مضادات الأكسدة الباطنية" بما في ذلك محتويات الجلوتاثيون، ومستوى نشاط الجلوتاثيون بيروكسيداز. وكشف الفحص الهستوباثولوجي لبطين قلب الجرذ أن العلاج بالدوكسوروبوسين تسبب في تحلل خلايا وتلف عضلة القلب. ومن ناحية أخرى، تسبب العلاج بالباكليتاكسل تغيرات معنوية في المؤشرات البوكيميائية ولكن بصورة أقل من تلك الناتجة من الدوكسوروبوسين منفرداً. لقد نتج عن تآلف الدوائين تفاقم تسمم القلب بغض النظر عن الفترة الزمنية الفاصلة بين إعطاء أي من الدوائين. كما ظهر أن إعطاء الباكليتاكسل بعد 30 دقيقة أو 24 ساعة من العلاج بالدوكسوروبوسين تسبب في تسمم القلب بصورة أكثر وضوحاً بالمقارنة مع التعاقب العكسي للدوائين. وقد تجلى هذا التفاقم بتغيرات أشد وضوحاً في مصل الدم وفي مؤشرات نسيج القلب التي تم قياسها. نستنتج من ذلك، أن الباكليتاكسل قد يؤدي إلى تفاقم تسمم القلب المستحث بالدوكسوروبوسين تعاونياً، وقد يكون التأثير أكثر شدة في تلك الجرذان التي تم علاجها بالباكليتاكسل بعد 24 ساعة من العلاج بالدوكسوروبوسين.

The higher incidence of cardiotoxicity of doxorubicin (DOX)/paclitaxel (PTX) combination compared with DOX alone remains to be a major obstacle against effective chemotherapeutic treatment. We investigated the effect of sequence and time of administration of both drugs on the severity of cardiotoxicity of the combination. Male Wistar rats were divided into seven groups. DOX was administered intraperitoneally (ip) at a single dose of 5 mg kg^{-1} every other 2 days, 2 doses per week for a total cumulative dose of 20 mg kg^{-1}. PTX was administered by an ip route at a dose of 20 mg kg^{-1} every other 2 days. Both drugs were injected either alone or sequentially in combination. In one case, DOX preceded PTX by 30 min or 24 h and in the other case, PTX preceded DOX by 30 min or 24 h. Cardiotoxicity was evaluated by both biochemical and histopathological examination, 48 hours after the last dose of DOX. DOX-induced cardiotoxicity was manifested by abnormal biochemical changes including marked increase in the activity of serum creatine phosphokinase isoenzyme (CK-MB), lactate dehydrogenase (LDH), glutathione

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peroxidase (GSH-Px) and aspartate aminotransferase (AST). Myocardial tissue from DOX treated rats showed significant increases in malondialdehyde (MDA) production and total nitrate/nitrite (NOx) level, parallel with depletion of "endogenous antioxidant reserve" including, GSH contents and GSH-Px activity level. Histopathological examination of ventricles of rat's heart revealed that DOX treatment produced myo-cytolysis and myocardial necrosis. On the other hand PTX treatment produced significant changes in the biochemical parameters but at a lower magnitude than those changes produced by DOX alone. Combination of both drugs resulted in aggravation of cardiotoxicity regardless the sequence and time interval between administration of either drug. Administration of PTX 30 min or 24 h after DOX treatment showed more pronounced cardiotoxicity compared with the reverse sequence. This exacerbation was manifested by much more pronounced changes in serum and cardiac tissue parameters measured. In conclusion, PTX might synergistically aggravate DOX-induced cardiotoxicity. The effect might be much more pronounced with those rats treated with PTX 24 h after DOX treatment.

Key words: Cardiotoxicity, Doxorubicin, Poclitaxel, Sequential.

Introduction

Doxorubicin (DOX) continues to be a mainstay chemotherapeutic agent, but cardiotoxicity remains to be a significant dose-limiting side effect requiring lifetime dosing limits (1). Although, the general phenomenon of cardiotoxicity is well known in human and animals, dose dependency and timing of DOX toxicity are often variable and difficult to predict. Many patients exhibit cumulative dose-dependent toxicity, but others experience acute life-threatening reactions or delayed cardiomyopathy manifesting months or years after the cessation of therapy (2).

Although oxidative chemistries have long been implicated in the etiology of DOX-induced cardiotoxicity, the putative reactive oxygen species (ROS) involved and/or the mechanism by which injury occurs remains far from being understood. A contemporary theory of the mechanism of DOX-induced cardiac dysfunction is based on tissue oxidation through increased cellular superoxide anion ($O_2^{\cdot -}$) formation.

The anthracycline ring structure of DOX has been shown to undergo both enzymatic and nonenzymatic single-electron redox cycling, liberating $O_2^{\cdot -}$ from molecular oxygen (3). Several flavoprotein reductases (e.g. cytochrome-P450 reductase and nitric oxide synthase) activate DOX-dependent redox cycling (4). The target organelle of DOX-induced toxicity in cardiomyocytes is the mitochondria, which accumulates DOX over time. In DOX-treated cardiomyocytes, the appearance of disorganized desmin and contractile filaments is related to detrimental alterations in the mitochondrial structure, in particular their position and trans-membrane potential (5). Mitochondrial enzymes (e.g. NADH dehydrogenase) have been shown to activate DOX to form the semiquinone radical and superoxide anion (6). Moreover, myocardial impairment caused by DOX may involve myocyte apoptosis presumably mediated by oxidative free radical formation (7). Thus, although the clinical importance of DOX-related cardiac dysfunction and toxicity is widely recognized, the precise cellular events have not been established, and the optimal therapeutic approaches for cardioprotection are not fully defined.

The taxanes have quickly been established as important chemotherapeutic agents in the armamentarium of drugs to treat breast cancer. Initial phase II findings of the concurrent doxorubicin-paclitaxel (PTX) combination resulted in substantial response rates, but at high cost. A much higher percentage of patients than expected developed anthracycline-induced cardiomyopathy (8). Paclitaxel (PTX) is an antimicrotubule agent induces mitotic block and apoptosis (9).

Stretch-induced arrhythmias were produced by transiently increasing the volume of a fluid-filled left ventricular balloon with a volume pump driven by a computer-controlled stepper motor. The probability of eliciting a stretch-induced arrhythmia increased in hearts treated with PTX, indicating the possible mode of arrhythmogenesis in patients receiving chemotherapy (10).

Markman *et al* (11) reviewed the experience of approximately 400 patients with gynecological malignancies who had been treated with PTX. The authors found that none of these patients had any clinical worsening of cardiac function following PTX treatment. Rose *et al* (12) studied 52 patients with metastatic breast cancer who were treated with

high-dose chemotherapy. Patients who received PTX had a slight decrease in left ventricular ejection fraction (LVEF) following therapy, they remained completely asymptomatic. Also, Gollerkeri *et al* (13) reported that PTX can be safely administered in patients with underlying cardiac dysfunction.

Combination of DOX with PTX is clinically effective but there are concerns regarding the higher incidence of cardiotoxicity of the combination compared with DOX alone. The mechanism of the increased toxicity is still unclear (14). Several reports demonstrate that PTX potentiates DOX-induced cardiotoxicity following DOX/PTX combination treatment. The authors attributed the potentiated DOX-induced cardiotoxicity to an increase in cardiac tissue concentration of the drug or its active metabolite doxorubicinol, and/or pharmacokinetic interference of DOX elimination by PTX, an effect that is highly dependent on the interval between administration of the drugs and the duration of PTX infusion (15, 16).

In the present study, we investigated the effect of sequential administration of DOX and PTX on cardiotoxicity.

Materials and Methods:

Age-matched, male Wister rats weighing 200 ± 20 g were used. The animals were housed in special cages and allowed free access to standard rat pellets and water *ad libitum.* The study adhered to the guidelines of the National Institutes of Health for experimental use of animals. The study was approved by our Institutional Review Board.

Animals were randomly allocated into seven groups of 10 rats each. The three control groups were treated with an intraperitoneal (ip) injection of either normal saline or doxorubicin 5 mg kg^{-1} (Adriablastina, Farmitalia Carloerba, Milan, Italy) every other 2 days, 2 doses per week for a total cumulative dose of 20 mg kg^{-1} that is approximately equivalent to the human cumulative dose of 900 mg m^{-2}. Paclitaxel was administered at a dose of 20 mg kg^{-1} every other 2 days (Taxol, Bristol-Myers Squibb Co., NJ, USA). The remaining groups were treated with a combination of both drugs. In one case, DOX preceded PTX by either 30 min or 24 h and in the other case, PTX preceded DOX by either 30 min or 24 h. Fourty eight hours after the last dose of DOX, animals were anaesthetized with ether and blood samples were taken by heart puncture. Serum was separated and used for measurement of indices of cardiotoxicity. Hearts were removed from the animals, washed with ice-cold saline, blotted with a piece of filter paper and divided into 2 halves. One half of each heart was homogenized (Biohomogenizer) in ice-cold bi-distilled water.

Ventricles of the second half of each heart were fixed in 10% neutral formalin, then embedded in paraffin, sectioned at 3 μm, stained with haematoxylin and eosin (H/E) and examined by light microscopy. The ventricle specimens were evaluated for typical histopathological features associated with DOX-induced cardiotoxicity (including, myocyte vacuolar degradation, necrosis of myofibers and interstitial fibrosis). Each specimen was scored for the degree of severity of histopathological changes. (A) Myocardial fiber swelling and interstitial oedema (1+); (B) dis-organization of myocardial fiber with or without fibroblastic proliferation (1+); (C) myo-cytolysis/necrosis of myocardial fibers (1+) and when no damage is noted (0). The above changes were judged as significant if seen in three or more high-power fields. The cardiomyopathy (CMY) severity scores were graded from zero to 3: 0 represents no CMY, 1 represents mild CMY, 2 represents moderate CMY and 3 or more represents sever CMY.

Serum creatine kinase isoenzyme (CK-MB) and lactate dehydrogenase (LDH) activities were measured kinetically at 340 nm according to standard methods using diagnostic kits from Randox Laboratories Ltd., UK. Serum glutathione peroxidase (GSH-Px) and aspartate aminotransferase (AST) activities were determined according to the methods of Paglia and Valentine (17) and Bergemeyer *et al* (18), respectively. Tissue total nitrate/nitrite (NOx), GSH levels as well as GSH-Px activity were determined according to the methods of Miranda *et al* (19), Ellman (20) and Paglia and Valentine (17), respectively. Tissue lipid peroxidation in terms of malondialdehyde (MDA) production levels was determined as thiobarbituric acid-reactive species according to the method of Ohkawa *et al* (21).

The statistical significance of the differences noted in the biochemical parameters was evaluated using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison test as a *post hoc* analysis. A p value of 0.05 or less was taken as the criterion for a statistically significant difference. For the histopathological changes observed, the statistical differences were evaluated by the Kruskal-Wallis one-way method of variance analysis.

Results

The effects of sequence and timing of administration of DOX and PTX on the measured biochemical markers presented in tables 1,2 & 3. The elevation of serum activities of cardiac isoenzymes, CK-MB and LDH is a well known quantitative index of compromised cell integrity, and also considered as an indicator of DOX-induced myocardial damage. DOX treated rats showed extensive elevation in serum CK-MB, LDH, GSH-Px and AST activity levels (3.9, 2.8, 1.4 and 2.3 fold changes, respectively) in comparison with saline treated controls ($p < 0.001$) (Table 1). Also, DOX treatment produced pronounced depletion of the cardiac tissue's "endogenous antioxidant reserve" (GSH and GSH-Px tissue levels) and elevation of MDA production and total NOx content levels (55 & 33% decrease and 155 & 70% increase, respectively) ($p < 0.001$) (Table 2).

Similarly, PTX treatment produced significant increases in rat's serum CK-MB, LDH, GSH-Px and AST activity levels by 1.8, 1.7, 1.12 and 1.5 fold changes, respectively ($p < 0.001$). In addition, cardiac tissue of PTX treated rats showed significant depletion in GSH and GSH-Px and increases in MDA production and NOx content levels (17 & 14 % decrease and 38 & 21 % increase, respectively) ($p < 0.001$) (Tables 1 & 2).

Combination of both drugs produced exacerbation of cardiotoxicity as compared with DOX treated group. This was manifested by significant elevation in cardiac markers (serum CK-MB & LDH) and serum GSH-Px activity ($p < 0.001$). Moreover, DOX/PTX combination treated rats demonstrated significant depletion of cardiac GSH-Px with rebound of the cardiac GSH contents and increases in lipid peroxidation in term of MDA production level and NOx content in comparison with those rats treated with DOX alone, depending on the sequance of administration ($p < 0.001$) (Tables 1 & 2).

When DOX treatment preceded PTX by 30 min and 24 h effect on exacerbation of DOX-induced cardiotoxicity was marked in comparison with those rats treated with DOX/PTX combination in a manner that PTX preceded DOX by 30 min and 24 h ($p < 0.001$) (Table 1 & 2).

Administration of DOX 24 h before PTX treatment produced much more pronounced changes in cardiac indices (CK-MB and LDH), serum GSH-Px and cardiac GSH-Px activities (10.5 & 11% increases and 14% decrease, respectively) and no significant changes in MDA production and NOx levels in the cardiac tissue in comparison with those rats treated with DOX, 30 min prior to PTX administration ($p < 0.001$). However, administration of PTX 24 h prior to DOX produced significant elevation in serum CK-MB and LDH activities ($p < 0.001$) (24.5 and 45.5% increases, respectively) and non-significant change in serum GSH-Px and AST levels in comparison with those rats treated with PTX 30 min prior to DOX ($p > 0.05$) (Table 1). Peroxidative alterations and NOx didn't show changes between groups treated with PTX 30 min or 24 h prior to DOX administration ($p > 0.05$) (Table 2). No significant change in cardiac NOx level between animals treated with either DOX alone or PTX, 30 min and 24 h prior to DOX administration ($p > 0.05$) (table 2).

Table1: Serum levels of creatine phosphokinase isoenzyme (CK-MB), lactate dehydrogenase (LDH), glutathione peroxidase (GSH-Px) and aspartate aminotransferase (AST) activities of rats treated with DOX/PTX combination.

Group	CK-MB $U\ L^{-1}$	LDH $U\ L^{-1}$	GSH-Px $U\ L^{-1}$	AST $U\ L^{-1}$
Control	248 ± 16	236 ± 22	1288 ± 76	154 ± 4.0
DOX	964 ± 124	651 ± 73	1809 ± 56	353 ± 25^{c}
PTX	456 ± 46	397 ± 54	1446 ± 86	234 ± 34
DOX 30 min → PTX	1506 ± 86	1058 ± 77^{a}	3873 ± 150	345 ± 32^{cde}
PTX 30 min → DOX	1104 ± 124	776 ± 61	3287 ± 519^{b}	322 ± 27^{df}
DOX 24 h → PTX	1661 ± 73	1544 ± 64	4297 ± 385	352 ± 49^{cf}
PTX 24 h → DOX	1375 ±92	1129 ± 128^{a}	3106 ± 303^{b}	319 ± 29^{ef}

Rats were injected ip with a single DOX dose (5 mg kg^{-1}) every other 2 days, 2 doses per week for a cumulative dose of 20 mg kg^{-1} either alone or 30 min and 24 h before or after PTX (20 mg kg^{-1}, ip every other 2 days). Values are the mean ± SD (n = 6-8). Statistical significance between means was analyzed using one-way ANOVA followed by the Tukey-Kramer multiple comparison test as a post hoc ($p < 0.001$). Means marked with the same superscript letters are not significantly different ($p > 0.05$).

Table 2: Effect of sequence and time interval between administration of both drugs on peroxidative alterations and total nitrate/nitrite of cardiac tissue of rats treated with DOX/PTX combination

Group	GSH μmol g^{-1}	GSH-Px U g^{-1}	MDA nmol g^{-1}	NOx μmol g^{-1}
Control	3.30 ± 0.137^{ab}	28.59 ± 2.77	321.5 ± 22.85	308.3 ± 19.83
DOX	1.50 ± 0.240	19.08 ± 1.63	820.6 ± 85.30^{a}	525.4 ± 42.26^{a}
PTX	2.75 ± 0.393^{c}	24.65 ± 2.36	442.3 ± 43.34	372.9 ± 26
DOX 30 min → PTX	3.16 ± 0.263^{acd}	14.85 ± 1.39	1026 ± 60^{b}	637 ± 41^{b}
PTX 30 min → DOX	3.21 ± 0.305^{bd}	17.00 ± 1.21^{a}	888.3 ± 47.38^{ac}	521.4 ± 17.65^{a}
DOX 24 h → PTX	3.21 ± 0.133^{d}	12.75 ± 1.94	1097 ± 105.71^{b}	632.1 ± 59.90^{b}
PTX 24 h → DOX	3.21 ± 0.195^{d}	16.67 ± 1.08^{a}	923.0 ± 63.16^{c}	511 ± 16.76^{a}

Details of the legend are as in table 1.

Table 3: Histopathological changes in the myocardium of rats treated with doxorubicin/paclitaxel combination: Effect of sequence of administration.

Group	n[a]	Cardiomyopathy score[b] Median	range
Control	8	0	
DOX	8	2.5	(2—3)[c]
PTX	8	1	(0—1)
DOX 30 min→ PTX	6	3.5	(3—4)[d]
PTX 30 min → DOX	7	2	(2—3)[c]
DOX 24 h → PTX	6	3.5	(3—4)[d]
PTX 24 h → DOX	6	2.5	(2—3)[c]

Treatment of rats was as in table 1.
Values are the median and values present in parenthesis represent the range. [a] Number of samples. [b] the cardiomyopathy (CMY) scores were graded from 0 to 3 by histopathologic study: 0 represent no CMY, 1 represents mild CMY, 2 represents moderate CMY and 3 or more represents sever CMY.
The significance of the differences between groups was analyzed by kruskal-wallis ANOVA ($p < 0.001$). Medians marked by the same superscript letters are not significantly different ($p > 0.05$)

The histopathological changes in the myocardium are reported in table 3 and fig. I. Qualitatively, DOX treatment produced myocytolysis and myocardial necrosis (Fig Ia). However, PTX treatment produced cytoplasmic vacuolation of the cardiac myocytes. Treatment with DOX in such way that it preceded PTX treatment by 30 min and 24 h produced marked myocardial swelling, interstitial oedema, inflammation , disorganization of myocardial fibers with fibroblastic proliferation and necrosis. Preceding PTX by DOX, 24 h apart produced extensive myocardial necrosis in comparison with those treated with DOX, 30 min prior to PTX administration (Fig. I b&c). However, preceding DOX treatment by PTX administration either by 30 min or 24 h produced non-significant

histopathological changes different from those rats treated with DOX alone (Table 3).

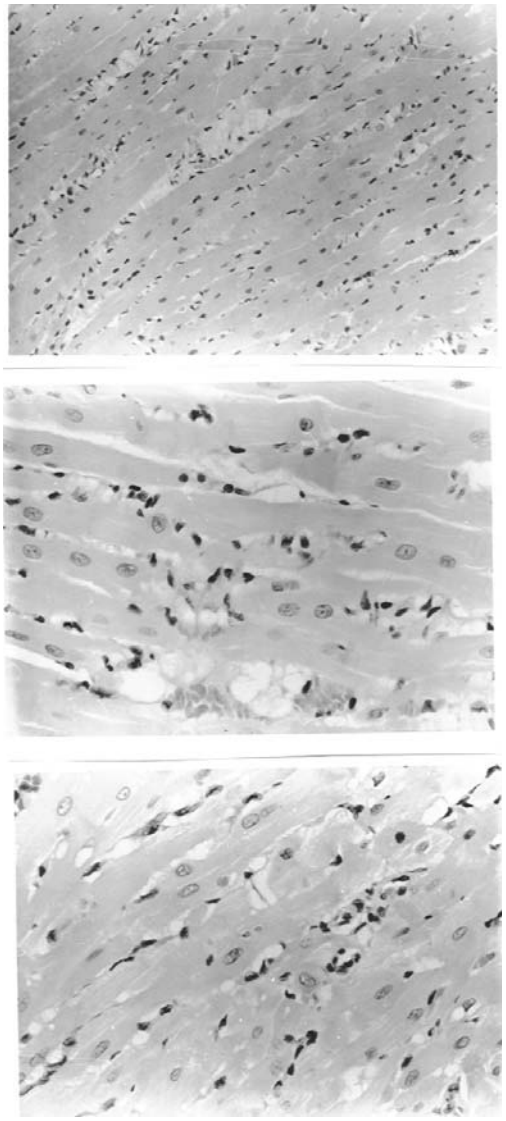

Fig. 1 a. Photomicrograph of myocardium section taken from a rat treated with DOX depicting marked interstitial oedema, mononuclear cell infiltration, myocardial fiber swelling and disorganization with peri-nuclear vacuolation. HE. X 200. **b**. Photomicrograph of a myocardium section taken from rat treated with PTX 24 h following DOX treatment showing swelling of the myocardial fibers with disorganization and extensive focal necrosis. HE. X 400. **c**. Photomicrograph of a myocardium section taken from a rat treated with DOX 30 min prior to PTX administration showing myocardial fiber swelling with necrosis and per-nuclear vacuolation. HE. X 400.

Discussion

In the present study we report that PTX potentiates DOX-induced cardiotoxicity in rats. Treatment of rats with DOX alone, at a cumulative dose of 20 mg kg^{-1} produced significant increases in serum CK-MB, LDH, GSH-Px and AST levels in comparison with saline treated controls. Also, DOX treatment depleted the cardiac "endogenous antioxidant reserve", manifested by GSH depletion and decrease in GSH-Px activity parallel with increases in MDA production and NOx content levels. Moreover, histopathological examination of ventricles of DOX treated rats revealed myocytolysis and necrosis characteristic to anthracyclines cardiotoxicity (Fig. I a). Our results are in accordance with those reported previously (22, 23).

Several plausible mechanisms have been reported for DOX-induced cardiomyopathy. Superoxide radicals generated by semiquinone intermediate ($DOX^{\cdot}$) upon one-electron reduction of O_2, release of Fe (II) in a stoichiometric manner from the mitochondrial aconitase, and reduction of iron catalyzed the formation of hydroxyl radical (24). Production of H_2O_2 due to DOX treatment resulted in activation of NF-Kappa β which acts as a pro-apoptotic in endothelial cells and myocytes (25). Moreover, DOX-induced iron signaling is regulated by the cell surface transferrin receptor (TFR) expression, intracellular oxidant levels and iron regulatory protein (IRP) (26). DOX can inactivate both IRP through a sequential action of doxorubicinol on IRP-1 or inactivation of IRP-2 by ROS (27). Also, DOX disturbs mitochondrial membrane and induces cytochrome C release into the cytoplasm in parallel with increase in caspase-activity (28).

Rats treated with PTX revealed marked increases in serum CK-MB, LDH, GSH-Px and AST levels. In addition, PTX treatment produced significant depletion of cardiac GSH level and GSH-Px activity and increases in MDA production level and NOx contents by a lower magnitude than those changes produced by DOX alone. Histopathologically, PTX treatment showed cytoplasmic vacuolation of the cardiac myocytes.

Treatment with the microtubule-interfering agent, PTX induces an increase in respiration and a

caspase-independent production of cytochrome oxidase mediated ROS by mitochondria which induces suicide pathways contributing to PTX cytotoxicity (29, 30). Also, PTX induces cytochrome C release in caspase-independent and permeability transition pore-dependent manner. Thus, PTX targets mitochondria upstream of caspase activation, early during the apoptotic process (29). Moreover, PTX plays a prominent role in the activation of the inducible NO pathway in response to inflammatory mediators (31). PTX processes the ability to induce NO production and increases TNF-$\propto$ and interleukin-1 secretion by macrophages via signal transduction pathway of PKC activation (31- 34). In addition, PTX enhances prostaglandin E_2 synthesis and increases COX-2 protein expression and COX-2 m-RNA levels (35). Increased COX-2 expression induced by PTX might play a role in oxidative alterations in the heart (36). Collectively, these explanations might support the mild cardiomyopathy induced by PTX treatment observed in the present study.

Regardless the sequence of administration, PTX has been shown to potentiate DOX-induced cardiotoxicity. This potentiation was manifested by more significant increases in cardiac markers (CK-MB and LDH) as well as serum GSH-Px and AST levels. The marked elevation in serum GSH-Px level could be attributed to massive cell destruction and/or excessive production of GSH-Px to counteract the oxidative stress induced by both DOX and PTX. Moreover, combination of both drugs together produced exacerbation of cardiac peroxidative alterations and NOx content with the rebound of cardiac GSH content. The GSH rebound could be attributed to increased consumption of GSH in the enzymatic and non-enzymatic detoxification of ROS generated by both drugs, which would, in turn, require enhanced gene expression of γ-glutamylcysteine synthase to increase the synthesis of GSH for maintaining a stable cellular level under the stress of DOX/PTX combination therapy (37). Exacerbation of cardiotoxicity of the combination was confirmed by the histopathological examination of the rat's myocardium.

Potentiation of DOX-induced cardiotoxicity by PTX could be attributed in part to NO. The increase in NO production produces an increase in myocardial aldose reductase activity, leading to higher lactate/pyruvate ratio (a measure of cytosolic NADH/NAD), lower tissue content of ATP and impaired cardiac function (38). Also, augmentation of DOX-induced cardiotoxicity could be attributed to the fact that both drugs have the capacity of inducing increase in ceramide production pathway. Ceramide is a known messenger of apoptosis (39-41). Both DOX and PTX induce NO production and high levels of endogenous myocyte-derived NO blunt myofilament Ca++ sensitivity (42).

In terms of serum cardiac damage indices as well as cardiac tissue oxidative indices and NO metabolites, administration of PTX 30 min and 24 h prior to DOX treatment exacerbated DOX-induced cardiotoxicity at a lower magnitude in comparison with the reverse sequence (30 min and 24 h post DOX). Moreover administration of PTX 24 h post DOX treatment produced the most dramatic exacerbation of the combination-induced cardio-toxicity.

The cardiodepressant and arrhythmogenic activity of PTX might be due to coronary vasoconstriction (43). Thus coronary vasocon-striction induced by PTX treatment prior to DOX might reduce cardiac muscle perfusion and the amount of DOX reaching the myocardium. This could contribute to lower cardiotoxicity of DOX/PTX combination, when PTX preceded DOX administration by 30 min and 24 h as compared with the reverse sequence of administration. In addition, ischaemic preconditioning of the cardiac muscle as a result of PTX-induced coronary vasoconstriction prior to DOX treatment might contribute for the lower aggressiveness of DOX-induced cardiotoxicity compared with the reverse sequence of administration.

On the contrary, combination of both drugs in which DOX preceded PTX is more cardiotoxic and this could be attributed to increase in the conversion of the cardiac accumulated DOX to DOX-ol by NADPH-dependent aldo/keto or carbonyl reductases via PTX-induced allosteric modulation of the reductases (44). Accumulation of DOX-ol in the cardiac myocytes may play an important role in the time-dependent development of DOX-induced ventricular dysfunction.

Cytochrome P-450 reductase is thought to be the major enzyme responsible for the one-electron reduction of DOX. Degradation of P-450 by lipid peroxidation is closely related to the oxidation of certain essential thiol groups located at the substrate binding site of the P-450 molecule. This could explain the lower cardiotoxicity of DOX/PTX

combination in which PTX preceded DOX by 30 min and 24 h. Lipid peroxidation induced by PTX might inactivate P-450 reductase (45, 46). This might reduce extensive conversion of the on going to be accumulated DOX to its semiquinone intermediate and attenuate DOX-induced cascade of cardiotoxicity. PTX releases Ca++ from an intracellular Ca++ stores. DOX treatment prior to PTX renders myocytes much more susceptible to agents that increase cytosolic Ca++, leading to depletion of ATP and eventually cell death. This might explain the exacerbated cardiotoxicity observed with those rats treated with PTX 30 min and 24 h post DOX treatment.

From the pharmacokinetic point of view, Sparano (16) reported that PTX interferes with DOX elimination, an effect that is highly dependent on the interval between administration of both drugs and the duration of PTX infusion. A sophisticated pharmacokinetic study showed that PTX enhances the non-linearity of DOX pharmacokinetics and significantly decreases the systemic clearance of both DOX and DOX-ol. The DOX/PTX interaction was found to be PTX-dose dependent, DOX concentration-dependent and may be the result of competition for elimination mechanisms (47). However, Platel *et al* (15), showed that the potentiation of DOX-induced cardiotoxicity by PTX is not related to an increase in tissue concentration of DOX or its active metabolite.

Combination of bolus DOX followed by 3 h infusion of PTX has high antitumor activity in patients with metastatic breast cancer. While PTX pharmacokinetics were not changed, there was a 30% and an 80% increase in the area under the curve (AUC0→24 h) for DOX and DOX-ol, respectively, when both drugs were administered 30 min instead of 24 h apart. Even when PTX was given 24 h after DOX, there was a rebound 240% increase in the plasma concentration of DOX-ol and this support our results (48).

Danesi *et al* (49), reported that in patients given PTX in combination with DOX, the peak plasma drug concentration (Cmax) of DOX increased significantly and drug clearance was reduced in the sequence PTX→DOX as compared with DOX→PTX. The schedule PTX→DOX was more toxic which might be non-cardiac toxicity as compared with DOX→PTX. Interestingly, the same author reported that 18 to 20% of congestive heart failure was observed in those patients treated with DOX followed by PTX.

In summary, PTX administration synergistically might potentiate DOX-induced cardiotoxicity in schedule-dependent manner. Preceding DOX by PTX might be less cardiotoxic combination in comparison with the reverse order of administration. The idea still warrants further investigation for the pharmacodynamic interaction of DOX/PTX combination at the myocytes level to define the exact mechanism of PTX-induced potentiation of DOX cardiotoxicity and the effect of different schedules on the cardiotoxicity of DOX/PTX combination.

References

1. Weinstein DM, Mihm MJ, Bauer JA. Cardiac peroxynitrite formation and left ventricular dysfunction following doxorubicin treatment in mice. J Pharmacol Exp Ther 2000;294:396-401
2. Singal PK. Subcellular effects of adriamycin in the heart: A concise review. J Mol Cell Cardiol 1987; 19:817-28.
3. Sarvazyan NA, Askari A, Huang WH. Effects of doxorubicin on cardiomyocytes with reduced level of superoxide dismutase. Life Sci 1995; 57:1003-10.
4. Vasquez-Vivar J, Martasex P, Hogg N, Masters BSS, Pritchard KA Jr and Kalyanaraman B. Endothelial nitric oxide synthase-dependent superoxide generation from adriamycin. Biochemistry 1997; 36:11293-7.
5. Konorev EA, Kennedy MC, Kalyanaraman B. Cell-permeable superoxide dismutase and glutathione peroxidase mimetics afford superior protection against doxorubicin-induced cardiotoxicity: the role of reactive oxygen and nitrogen intermediates. Arch Biochem Biophys 1999; 368:421-8.
6. Sawyer DB, Fukazawa R, Arstall MA, Kelly RA. Daunorubicin-induced apoptosis in rat cardiac myocytes is inhibited by dexrazoxane. Circ Res 1999; 84:257-65.
7. Kotamraju S, Konorev EA, Joseph J, Kalyanaraman B. Doxorubicin-induced apoptosis in endothelial cells and cardiomyocytes is ameliorated by nitrone spin traps and ebselen. Role of reactive oxygen and nitrogen species. J Biol Chem 2000; 275:33585-92.
8. Valero V, Perez E, Dieras V. Doxorubicin and taxane combination regimens for metastatic breast cancer: Focus on cardiac effects. Semin Oncol 2001; 28(4 Suppl 12):15-23.
9. Andre N, Braguer D, Brasseur G, Goncalves A, Lemesle-Meunier D, Guise S, Jordan MA, Briand C. Paclitaxel induces release of cytochrome C from mitochondria isolated from human neuroblastoma cells. Cancer Res 2000; 60(19):5349-5353.
10. Parker KK, Taylor LK, Atkinson JB, Hansen DE, Wikswo JP, Atkinson B. The effects of tubulin-binding agents on stretch-induced ventricular arrhythmias. Eur J Pharmacol 2001; 417:131-40.
11. Markman M, Kennedy A. Webster K, Kulp B, Petreson G, Belinson J. Paclitaxel administration to gynecologic cancer

patients with major cardiac risk factors. J Clin Oncol 1998; 16:3483-5.
12. Rose M, Lee FA, Gollerkeri A, d'Andrea E, Psyrri A, Bdolah-Abram T, *et al*. The feasibility of high-dose chemptherapy in breast cancer patients with impaired left ventricular function. Bone Marrow Transplant 2000; 26:133-9.
13. Gollerkeri A, Harrold L, Rose M, Jain D, Burtness BA. Use of paclitaxel in patients with pre-existing cardiomyopathy: A review of our experience. Int J Cancer 2001; 93:139-41.
14. Colombo T, Parisi I, Zucchetti M, Sessa C, Goldhirsch A, D'Incalci M. Pharmacokinetic interaction of paclitaxel, docetaxel and their vehicles with doxorubicin. Ann Oncol 1999;10:391-5.
15. Platel D, Pouna P, Bonoron-Adele S, Robert J. Preclinical using the model of isolated perfused rat heart. Toxicol Appl Pharmacol 2000; 163:135-40.
16. Sparano JA. Doxorubicin/taxane combinations: Cardiac toxicity and pharmacokinetics. Semin Oncol 1999; 26(3 Suppl 9):14-9.
17. Paglia DE, Valentine WN. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J Lab Clin Med 1967; 70:158-69.
18. Bergemeyer HU, Scheibe P, Wahlefeld AW. Optimization of methods for aspartate aminotransferase and alanine aminotransferase. Clin Chem 1978; 24:58-73.
19. Miranda KM, Espey MG, Wink DA. A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. Nitric Oxide 2001; 5:62-71.
20. Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys 1959; 82:70-7.
21. Ohkawa H, Ohishi N, Yagi K. Assay of lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979; 95:351-8.
22. Cao Y, Kennedy R, Klimberg VS. Glutamine protects against doxorubicin-induced cardiotoxicity. J Surg Res 1999; 85:178-82.
23. Saad SY, Najjar TA, Al-Rikabi AC. The preventive role of deferoxamine against acute doxorubicn-induced cardiac, renal and hepatic toxicity in rats. Pharmacol Res 2001; 43:211-8.
24. Vasquez-Vivar J, Kalyanaraman B and Kennedy MC . Mitochondrial aconitase is a source of hydroxyl radical. An electron spin resonance investigation. J Biol Chem 2000; 275:14064-9.
25. Wang S, Kotamraju S, Konorev E, Kalivendi S, Joseph J, Kalyanaraman B. Activation of nuclear factor kappa β during doxorubicin-induced apoptosis in endothelial cells and myocytes is pro-apoptotic: The role of hydrogen peroxide. Biochem J 2002; 367:729-40.
26. Kotamraju S, Chitambar CR, Kalivendi SV, Joseph J, Kalyanaraman B. Transferrin receptor-dependent iron uptake is responsible for doxorubicin-mediated apoptosis in endothelial cells: role of oxidant-induced iron signaling in apoptosis. J Biol Chem 2002; 277:17179-87.
27. Minotti G, Ronchi R, Salvatorelli E, Menna P, Cairo G. Doxorubicin irreversibly inactivates iron regulatory protein 1 and 2 in cardiomyocytes: Evidence for distinct metabolic pathways and implications for iron-mediated cardiotoxicity of tumuor therapy. Cancer Res 2001; 61:8422-8.
28. Childs AC, Phaneuf SL, Dirks AJ, Phillips T, Leeuwenburgh C. Doxorubicin treatment in vivo causes cytochrome C release and cardiomyocyte apoptosis, as well as increased mitochondrial efficiency, superoxide dismutase activity and bcl-2 : bax ratio. Cancer Res 2002; 62:4592-8.
29. Andre N, Carre M, Brasseur G, Pourroy B, Kovacic H, Briand C, Braguer D. Paclitaxel targets mitochondria upstream of caspase activation in intact human neuroblastoma cells. FEBS Lett 2002; 532:256-60.
30. Varbiro G, Veres B, Gallyas FJr, Sumegi B. Direct effect of taxol on free radical formation and mitochondrial permeability transition. Free Radic Biol Med 2001; 31:548-58.
31. Kirikae T, Ojima I, Fuero-Oderda C, Lin S, Kirikae F, Hashimoto M, Nakanoc M. Structural significance of the acyl group at the C-10 position and the A ring of the taxane core of paclitaxel for inducing nitric oxide and tumor necrosis factor production by murine macrophages. FEBS Lett 2000;478:221-6.
32. Pae HO, Jun CD, Yoo JC, Kwak HJ, Lee SJ, Kook YA, Park RK, Chung HT. Enhancing and priming of macrophages for superoxide anion production by taxol. Immunopharmacol Immunotoxicol 1998; 20:27-37.
33. Marczin N, Jiilling T, Papapetroppouulos A, Go C, Catravas JD. Cytoskeleton-dependent activation of the inducible nitric oxide synthase in cultured aortic smooth muscle cells. Br J Pharmacol 1996; 118:1085-94.
34. Jum CD, Choi BM, Kim HM, Chung HT. Involvement of protein kinase C during taxol-induced activation of murine peritoneal macrophages. J Immunol 1995; 154:6541-7.
35. Subbaramaiah K, Hart JC, Norton L, Dannenberg AJ. Microtubule-interfering agents stimulate the transcription of cyclooxygenase-2. Evidence for involvement of ERK ½ and p38 mitogen-activated protein kinase pathways. J Biol Chem 2000; 275:14838-45.
36. Kim JW, Baek BS, Kim YK, Herlihy JT, Ikeno , Yu BP, Chung HY. Gene expression of cyclooxygenase in the aging heart. J Gerontol A Biol Sci Med Sci 2001; 56:B350-5.
37. Yin, Wu H, Cheen Y, Kang J. Induction of antioxidants by adriamycin in mouse heart. Biochem Pharmacol 1998; 56:87-93.
38. Hwang YC, Sato S, Tsai JY, Yan S, Bakr S, Zhang H, Oates PJ, Ramasamy R. Aldose reductase activation is a key component of myocardial response to ischemia. FASEB J 2002; 16:243-5.
39. Kawase M, Watanabe M, Kondo T, Yabu T, Tagguchi Y, Umehara H, Uchiyama T, Mizuno K, Okazaki T. Increase of ceramide in adriamycin-induced HL-60 cell apoptosis: detection by a novel anti-ceramide antibody. Biochim Biophys Acta 2002; 1584:104-14.
40. Charles AG, Han TY, Liu YY, Hansen N, Giuliiano AE, Cabot MC. Taxol-induced ceramide generation and apoptosis in human breast cancer cells. Cancer Chemother Pharmacol 2001; 47:444-50.
41. Feuerstein GZ. Apoptosis -new opportunities for novel therapeutics for heart diseases. Cardiovascular Drugs Ther 2001; 15:553-4.
42. Brunner F, Andrew P, Wolkart G, Zechner R, Mayer B. Myocardial contractile function and heart rate in mice with myocyte-specific overexpression of endothelial nitric oxide synthase. Circulation 2001: 104:3097-102.
43. Alloati G, Penna C, Gallo MP, Levi RC, Bombardelli E, Appendino G. Differential effects of paclitaxel and derivatives on guinea pig isolated heart and papillary muscle. J Pharmacol Exp Ther 1998; 284:561-7.

44. Minotti G, Saponiero A, Licata S, Menna P, Calafiore AM, Teodori G, Gianni L. Paclitaxel and docataxel enhance the metabolism of doxorubicin to toxic species in human myocardium. Clin Cancer Res 2001; 7:1511-5.
45. Garner A, Paine MJ, Rodriguez-Crespo I, Chinjje EC, Ortiz De Montellanoo P, Stratford IJ, Tew DG, Wolf CR. Nitric oxide catalyze the activation of redox cycling and bioreductive anticancer agents. Cancer Res 1999; 59:1929-34.
46. Lee DW, Yu BP. Microsomal cytochrome P-450 degradation by in vitro lipid peroxidation. J Nutr Health Aging 1998; 2: 162-6.
47. Holems FA, Rowinsky EK. Pharmacokinetic profiles of doxorubicin in combination with taxanes. Semin Oncol 2001; 28(4 Suppl 12):8-14.
48. Moreira A, Lobato R, Morais J, Silva S, Ribeiro J, Figueira A, Vale D, Sousa C *et al*. Influence of the interval between the administration of doxorubicin and paclitaxel on the pharmacokinetics of these drugs in patients with locally advanced breast cancer. Cancer Chemother Pharmacol 2001; 48:333-7.
49. Danesi R, Conte PF, Del Tacca M. Pharmacokinetic optimization of treatment schedules for anthracyclines and paclitaxel in patients with cancer. Clin Pharmacokinet 1999; 37:195-211.