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Oxidative stress induced in rat liver by anticancer drugs doxorubicin, paclitaxel and docetaxel

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ABSTRACT

Purpose: Oxidative stress generated by anticancer drugs in non-targeted tissues, is considered as a significant factor responsible for their severe side effects, e.g. cardiotoxicity, neurotoxicity and hepatotoxicity. Lack of data on the effect of concurrent administration of commonly used anticancer drugs: doxorubicin (DOX), paclitaxel (PTX) and docetaxel (DTX) on normal tissue, prompted us to examine the markers of oxidative stress in the liver of rats treated with these drugs.

Material/Methods: Male Wistar rats of average weight 200 g were injected intraperitoneally (i.p.) with 10 mg/kg of body weight (b.w.) of DOX, PTX and DTX. The drugs were given alone or in combinations DOX+taxane. The activities of superoxide dismutase (SOD), catalase (CAT), low molecular weight and total thiols and thiobarbituric acid-reactive substances (TBARS) were estimated.

Results: Combination of two drugs generated greater changes than single agents. Concurrent administration of DOX and PTX increased SOD activity and TBARS, decreased the amount of low molecular weight and total thiols, but did not cause any changes in the activity of catalase. Combination of DOX and DTX induced similar changes except for the activity of catalase, which decreased after the treatment. Of the three drugs only DTX significantly decreased the activity of SOD. However, both taxanes increased the activity of catalase. Although a decrease in concentration of –SH groups, depletion of glutathione and an increase of TBARS were observed after treatment with single drugs, the changes were not statistically significant.

Conclusion: Concurrent administration of DOX and taxane induced enhanced oxidative stress in comparison to single drugs, which suggests their synergistic prooxidant mode of action in liver.

Key words: doxorubicin, paclitaxel, docetaxel, oxidative stress, rat liver

INTRODUCTION

Chemotherapy is one of the principal methods employed in the treatment of several types of cancer, allowing for use of a combination of different types of antineoplastic drugs to increase its efficiency [1]. Both cancer and chemotherapy-associated complications, cause, however, substantial mortality. The objective of chemotherapy is to eliminate exclusively tumour cells. Most of the antineoplastic agents act, however, non-specifically, harming both malignant and normal cells. Toxicity of anticancer drugs toward normal tissues has a significant impact on the condition and treatment

outcome of patients undergoing chemotherapy. Severe side effects caused by commonly used anticancer drugs often limit the efficiency of chemotherapy due to the necessity of reduction of drug doses or discontinuation of treatment. Cytotoxic action of anticancer drugs may increase as a result of the use of combined therapy employing more than one drug, which also applies to anthracyclines and taxanes. The combinations of taxanes with DOX are widely used to treat advanced and metastatic breast cancer and other solid tumours. These drugs are highly effective, do not exhibit cross-resistance and have different toxicity profiles [2]. Similarly to other antineoplastic agents they cause numerous side effects. Some recent papers have been devoted to the liver damage caused by these drugs [3].

Hepatotoxicity is associated with impaired liver function, caused by exposure to a drug or other factors severely impairing its function [2]. The liver is responsible not only for many crucial functions within the body, but also for biotransformation of drugs, their detoxification and conversion into the forms that can be readily eliminated from the body. Interaction of anthracyclines, e.g. doxorubicin with DNA is considered as the main mechanism of their toxicity, both in cancer and normal cells. Anthracycline drugs are effective inhibitors of the activity of topoisomerase II or I. Doxorubicin is widely applied in chemotherapy, but its use is limited by the high risk of cardiomyopathy and congestive heart failure development [4]. Cytotoxicity of anthracyclines is also associated with the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [5]. *In vivo* doxorubicin undergoes reduction to a semiquinone-free radical by microsomal and nuclear enzymes. Molecule of O_2 can accept an electron from semiquinone, which results in generation of superoxide anion radical. Doxorubicin can also bind ionic iron (Fe^{3+}). This complex is highly toxic to membrane lipids, proteins and DNA. The drug can also bind to the DNA and uncoil the double-stranded helix with generation of free radicals and DNA damage [6]. Cytosolic fraction of doxorubicin may be converted to doxorubicinol by NADPH-dependent aldo-/keto- or carbonyl reductases. This metabolite inhibits several membrane ATP-ases and isometric contraction of heart muscle [7].

Taxanes are mitotic inhibitors showing appreciable anticancer activity against breast and lung cancers. Taxanes come from the bark extract of the Pacific yew, *Taxus brevifolia*. The principal mechanism of their action is a disruption of microtubule function (inhibition of microtubule depolymerization) through stabilizing GDP-bound tubulin in the microtubule [8].

The available literature does not provide much information about the liver damage caused by taxanes (docetaxel and paclitaxel). Dose reduction is recommended for these drugs in patients with liver dysfunction because of the higher risk for neutropenia, mucositis, and treatment-related death. Vaclavikova *et al.* [9] indicated that, in contrast to paclitaxel, the rat, human, pig, and minipig microsomes formed the same metabolites of docetaxel, with a hydroxydocetaxel being the main product.

Despite numerous studies on mitochondrial ROS formation by taxanes, their mode of action still remains to be elucidated [10, 11]. Varbiro *et al*. [12] showed that PTX displayed mainly direct mitochondrial effect, induced mitochondrial permeability transition and ROS formation [10], and probably docetaxel has a similar mechanism of action.

In the present study, we investigated changes in some of the parameters of oxidative stress (low molecular weight thiols - mainly glutathione, and total thiols, TBARS) and the activity of antioxidant enzymes (catalase and superoxide dismutase) in liver tissue of rats treated with doxorubicin, paclitaxel, docetaxel or combination of antracycline with taxane.

MATERIAL AND METHODS

Animals

Two months old 180 – 220 g male Wistar rats of outbred strain Imp:WIST were obtained from Nofer Institute of Occupational Medicine in Lodz. Animals were kept under standard conditions with free access to pellet diet and clean drinking water.

All experiments were performed according to the guidelines of the European Community for the Use of Experimental Animals (L358-86/609/EEC) and the Guiding Principles in the Use of Animals in Toxicology (1989) and were approved by the Local Ethics Committee for Animal Experimentation in Lodz (2/LB357/2007).

Experimental setup

Rats were divided into 6 groups of 6 animals each. Tested anticancer drugs, suspended in 5% glucose were administered alone (10 mg/kg body weight (b.w.)) or in combination (DOX and taxanes, 10 mg/kg b.w. each) as a single 1 ml injection. Rats in group I received 5% glucose only and served as a negative control. Rats in groups II, III and IV were treated with doxorubicin, paclitaxel and docetaxel, respectively. Rats in group V and VI received two drugs injected simultaneously: doxorubicin and paclitaxel (group V) or doxorubicin and docetaxel (group VI).

On the fourth day after injection, the animals were anaesthetized and sacrificed by cervical decapitation. We applied this time point on the basis of our pilot study and the data obtained by other authors showing that maximal changes in lipid peroxidation and antioxidant enzymes appear within 3-5 days after injection of a single dose of doxorubicin or other related drugs [13-15].

Livers were immediately excised, washed with physiological saline, frozen on solid CO_2 and stored at -80°C until analysis. 10% homogenate was prepared in 1.15% KCl

on ice (1g tissue and 9 ml KCl), centrifuged at 3000 x g for 10 min at 4°C and the supernatant was used for biochemical assays.

Biomarkers of oxidative stress

Superoxide dismutase activity

Activity of SOD in liver homogenate was assayed by the indirect adrenaline spectrophotometric method of Misra and Fridovich [16], based on the ability of SOD to inhibit the autoxidation of adrenaline to adrenochrome at alkaline pH. Measurements were performed at 480 nm.

Catalase activity

Catalase activity was estimated by the method of Aebi [17], using hydrogen peroxide as a substrate. The method is based on the decomposition of hydrogen peroxide, which is indicated by the decrease in absorbance at 240 nm.

Low molecular weight thiols

The amount of low molecular weight thiols (glutathione, cysteine, homocysteine, etc.) was determined by the Ellman method [18]. Homogenate samples were precipitated with trichloroacetic acid (TCA) and the protein precipitate was removed by centrifugation. Further analysis was performed analogically to that used for determination of total thiol groups. Concentration of low molecular weight thiols was calculated from the standard curve for reduced glutathione and expressed as nmol/mg protein.

Total thiol groups

Concentration of –SH groups was estimated according to the Ellman method [18]. Following the reaction between Ellman's reagent with thiol groups, optically active cation was formed and its absorbance was measured at 412 nm. Optical activity of samples at this wavelength was measured before the addition of Ellman's reagent and subtracted. The concentrations of thiols were calculated from the calibration curve obtained for reduced glutathione (0-2 mM range of concentrations) as a standard. Results were expressed as mmol/mg protein.

Lipid peroxidation

Lipid peroxidation was evaluated on the basis of production of TBARS calculated from the 532 nm absorbance using an extinction coefficient of 156 mM^{-1} cm^{-1} [19].

Protein concentration

Protein concentration was determined with Folin reagent according to the spectrophotometric method of Lowry *et al* [20].

Statistical analysis

Statistical analysis included calculation of means ± SD in each group. The significance of differences was estimated by the Mann-Whitney U-test.

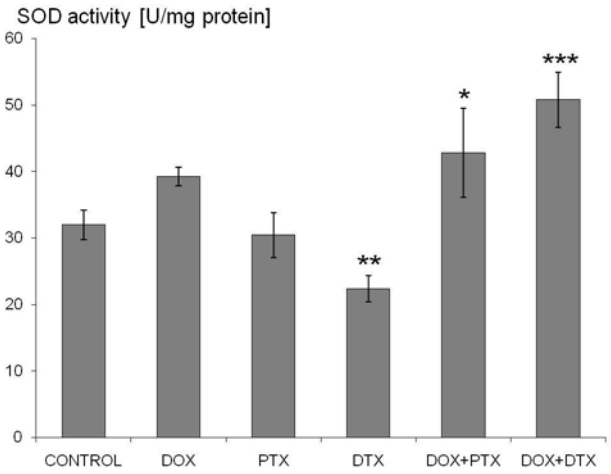
RESULTS

No animal deaths were observed in the course of the experiments.

The activities of superoxide dismutase (EC. 1.15.1.1) and catalase (EC. 1.11.1.6) are essential biomarkers of induction of oxidative stress in the liver tissue. SOD is an enzyme which catalyses the dismutation of superoxide anion to hydrogen peroxide and oxygen, while the role of catalase is the decomposition of hydrogen peroxide (H_2O_2) to water and oxygen and protection of cells against the toxic effects of ROS. Changes in SOD and CAT activities, induced in rat liver by treatment with the investigated anticancer drugs or their combinations (doxorubicin with paclitaxel/docetaxel), are presented on *Figure 1* and *Figure 2*, respectively.

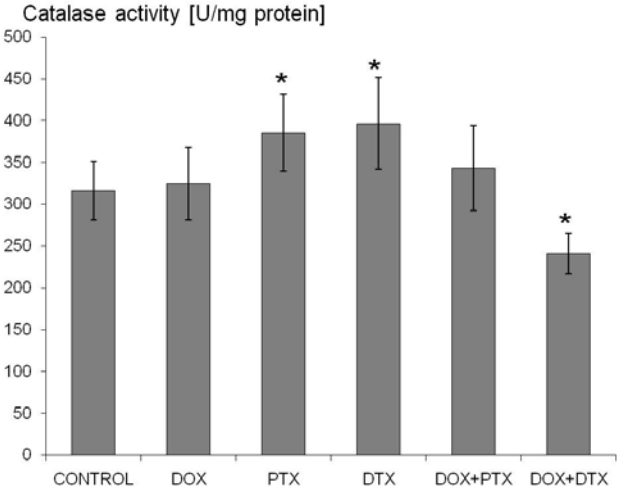
We observed a significant decrease in the activity of SOD only in the liver of rats injected with docetaxel ($p<0.002$). Although there were also small changes in the activity of this enzyme after doxorubicin (increase) or paclitaxel (decrease) treatment, the changes were not statistically significant. The effect of doxorubicin was enhanced by the addition of taxane. SOD activity statistically significantly increased

Figure 1. **Superoxide dismutase activity in rat liver after treatment with anticancer drugs doxorubicin (DOX), paclitaxel (PTX), docetaxel (DTX) or their combination (DOX with PTX or DOX with DTX).**



*Results are expressed as mean ± S.D. for 5 animals in each group. Statistical significance in comparison to control group (rats injected with a vehicle only, 5% glucose) is marked on the graph with stars (*p<0.05, **p<0.002, ***p<0.0002). Statistical significance of other comparisons is listed below:*
DOX + DTX vs. DOX p<0.002
DOX + DTX vs. DTX p<0.0002
DOX + PTX vs. PTX p<0.001

Figure 2. **Catalase activity in rat liver after treatment with anticancer drugs doxorubicin (DOX), paclitaxel (PTX), docetaxel (DTX) or their combination (DOX with PTX or DOX with DTX).**



*Results are expressed as mean ± S.D. for 6 animals in each group. Statistical significance in comparison to control is marked with stars (*p<0.05). Statistical significance of other comparisons is: DTX vs. DOX + DTX p<0.0002.*

when doxorubicin was applied in conjunction with paclitaxel or docetaxel. Changes were markedly higher when docetaxel was employed and statistically significant for both untreated controls and rats treated with any of the single drugs included in the combination.

In groups of animals injected with a single drug (groups II, III, IV), significant changes in CAT activity in comparison to untreated controls (an increase) were observed for taxanes only ($p<0.05$). Doxorubicin administered either alone or in combination with PTX did not affect catalase activity. However, DOX used in combination with docetaxel caused a statistically significant decrease in CAT activity in comparison to both the control group ($p<0.05$) and rats receiving docetaxel alone (group IV) ($p<0.0002$).

Results obtained for other biomarkers of oxidative stress (low molecular weight and total thiol groups and TBARS) are summarized in *Table 1.*

Significant changes in the pool of reduced glutathione were found only in animals treated with paclitaxel or paclitaxel in combination with doxorubicin (group III and V) ($p<0.05$). Although a decrease in low molecular weight thiols (mainly reduced glutathione) content was observed in other experimental groups (groups II, IV and VI), the changes were not statistically significant.

Results obtained for concentration of total thiol groups were largely correlated with changes observed for reduced glutathione, which suggests its appreciable participation in the observed changes. Amount of total –SH groups in the liver of rats treated with a combination of doxorubicin with paclitaxel (group V) was significantly higher in comparison to untreated controls and rats receiving doxorubicin or paclitaxel as a single agent (groups II and III). At the same time no differences in thiols between the experimental groups treated with DTX or DOX-DTX (groups IV and VI) were found.

Similar to changes in low molecular weight thiols and total thiol pools statistically significant changes in lipid peroxidation, estimated on the basis of the amount of produced

Table 1. **Concentration of low molecular weight thiols, total thiol groups and TBARS in liver of rats treated with doxorubicin (DOX), paclitaxel (PTX), docetaxel (DTX) or their combinations (DOX with PTX or DOX with DTX). Each value is expressed as mean ± S.D. for 6 animals in each group.**

Treatment groups	low molecular weight thiols (nmol/mg protein)	total thiols (mmol/mg protein)	TBARS (nmol/mg protein)
statistical significances in comparison with control: *p<0.05 **p<0.001 ***p<0.0002			
Control	4.77 ± 0.46	0.89 ± 0.03	0.074 ± 0.006
DOX	3.68 ± 0.29	0.73 ± 0.04	0.083 ± 0.004
PTX	1.83 ± 0.21*	0.76 ± 0.05	0.100 ± 0.010
DTX	2.75 ± 0.31	0.93 ± 0.04	0.100 ± 0.009
DOX + PTX	1.90 ± 0.20*	1.13 ± 0.06**	0.241 ± 0.023***
DOX + DTX	2.19 ± 0.12	1.05 ± 0.05	0.264 ± 0.024***
statistical significances between different groups			
DOX vs. DOX+PTX	p <0.01	p <0.0002	p <0.0002
DOX vs. DOX+DTX	n.s.	p <0.0002	p <0.0002
PTX vs. DOX+PTX	n.s.	p <0.0002	p <0.0002
DTX vs. DOX+DTX	n.s.	n.s.	p <0.0002

TBARS, were found only in the liver of rats treated with a combination of two drugs – doxorubicin with paclitaxel or doxorubicin with docetaxel (groups V and VI) ($p<0.0002$). None of the investigated drugs, injected as a single agent, induced noteworthy changes of lipid peroxidation in the liver of treated animals.

DISCUSSION

Taxanes, paclitaxel and docetaxel, are microtubule active drugs and one of the most potent cancer chemotherapeutics. Both of these drugs are employed in the therapy of a variety of human tumours, including ovarian carcinomas, breast and lung cancers [21-23]. Moreover, paclitaxel and docetaxel maintain substantial antitumour activity in anthracycline-resistant breast cancer [24]. Taxanes are also used in combined chemotherapy, which is superior to single drug use. PTX or DTX in conjugation with DOX are successfully used in the treatment of early, locally advanced and metastatic breast cancer [25-27]. Unfortunately, there is also a dark side of this therapy as taxanes can enhance toxicity of doxorubicin, e.g. cardiotoxicity, neurotoxicity, hepatotoxicity, nephrotoxicity and others. Combinations of DOX with taxanes appeared to be more toxic than DOX alone. Colombo *et al.* [28], have found that concentration of DOX in the heart, liver and kidney tissue in mice treated with DOX in conjunction with paclitaxel or docetaxel was significantly higher than in mice treated with DOX alone. The authors presented a hypothesis according to which cardiotoxicity induced by the combination of doxorubicin with taxanes includes the formation of ROS and may probably be correlated with drug retention in the neoplastic tissue. Moreover, taxanes increase conversion of doxorubicin to the more toxic metabolite doxorubicinol by NADPH-dependent aldo-/keto- or carbonyl reductases, which may contribute to the enhancement of anthracycline cardiotoxic effects [7]. Doxorubicinol production, among others, leads to the high incidence of congestive heart failure.

Generation of oxidative stress by doxorubicin has been proved to play a significant role in toxicity of this drug [10, 11]. ROS, induced by DOX treatment, are considered as one of the main reasons for cardiotoxic effects. It is associated with low activity of antioxidant system in cardiomyocytes [29, 30]. Application of DOX in the long term clinical treatment leads to cardiotoxicity and congestive heart failure, which is often lethal [31].

Taxanes, in contrast to doxorubicin, for a long time were not considered as prooxidative acting agents, but an increasing number of experimental data show that these anticancer drugs can induce considerable oxidative stress inside the cell [12]. Numerous studies demonstrated the importance of ROS production for taxane cytotoxicity *in vitro* and *in vivo* [32, 33]. Evidence for the involvement of ROS in paclitaxel cytotoxicity is steadily increasing for most, though not all, cellular models [34, 35]. Oxidative stress has been suggested to play a role in the antitumor effects of PTX. Cell lines with higher total antioxidant capacity have been found to be more resistant to paclitaxel cytotoxicity [36, 37]. Moreover, antioxidants, such as thiols (N-acetylcysteine, NAC), catalase and superoxide dismutase, inhibited cytotoxicity of PTX [38]. These results show that cytotoxicity of PTX in cancer cells may be mediated by the production of ROS, e.g. H_2O_2.

It has been found that resistance to paclitaxel is proportional to cellular total antioxidant capacity [39]. Compounds that decrease ROS level can also suppress PTX cytotoxicity. In contrast – increase of ROS level by inhibition of SOD or glutamylcysteine synthase enhanced PTX cytotoxicity [39]. Interference in microtubule dynamics, which is typical for taxane mechanism of action, is known for disruption of redox signalling, which can cause activation of NADPH oxidase and the production of intracellular ROS [40].

Paclitaxel was found to increase the production of hydroperoxides and to generate oxidative stress in human cancer cells [41, 42]. The drug increases the level of superoxide, hydrogen peroxide, nitric oxide and oxidative DNA adducts [38]. Hadzic *et al.* [38] showed that treatment of T47D and MDA-MB231 human breast cancer cells with PTX leads to increases in parameters of oxidative stress such as H_2O_2 and GSSG. Other study suggested that the accumulation of peroxides is an early and decisive step for paclitaxel-induced cancer cell death by apoptosis [41]. PTX can directly affect free radical formation and mitochondrial membrane polarization [12, 39].

Oxidative stress, induced by PTX treatment can participate in toxicity of this drug toward non-targeted tissues. Preclinical results suggested that genes, encoding antioxidant enzymes, can affect PTX neurotoxicity [43]. Paclitaxel has been also shown to induce hepatoxicity [44]. Ohlman *et al.* [45] reported on the lethal course of a patient receiving low-dose, weekly docetaxel who developed acute liver failure.

We did not observe markedly higher level of TBARS in the liver of rats treated with DOX and taxanes (groups V and VI) in comparison to untreated controls. Increase of TBARS after DOX treatment was reported by Kalender *et al.* [30] and Deepa *et al.* [46], who also used rats in their studies. Higher level of TBARS concentration after docetaxel treatment of rats was also found [47].

In our experimental model, doxorubicin injected alone and in combinations with taxanes induced an increase of activity of SOD. Similar changes in the liver of rats treated with DOX were also observed by other authors [48]. At the same time we have found a decrease in SOD activity in the liver of rats receiving docetaxel. These data are in agreement with *in vitro* study on hepatocytes treated with docetaxel [49]. On the basis of our results we can conclude that DOX and taxanes can act synergistically and thus, can possibly trigger more significant liver damage when used in combination.

Taxanes, applied as single agents, increased activity of

catalase in rat liver, in contrast to DOX, which did not cause any changes. This is inconsistent with the study by Kalender *et al.* [30], where an increase in catalase activity in the rat liver after DOX injection was found. Both taxanes in concurrent administration with DOX, behaved differently and changes in CAT activity (a decrease) were noted only for combination of docetaxel with doxorubicin.

We observed a depletion of low molecular weight thiols in the rat liver after treatment with either of the studied drugs, but statistically significant results were obtained only for combination DOX-PTX. Similar results were obtained for total thiols.

Low molecular weight thiols such as glutathione, cysteine, homocysteine and other were determined. However, the concentrations of cysteine and homocysteine in the liver tissue are very low in comparison to glutathione [50]. Reduced glutathione (GSH) is the main intracellular nonprotein thiol and its redox status is critical for various biological events, including transcriptional activation of specific genes and modulation of redox-regulated signal transduction [51]. GSH has been implicated in the regulation of cellular proliferation and apoptosis, immune modulation and inflammatory response [52]. The balance between reduced and oxidized levels of GSH depends on the redox status of the cell as well as *de novo* GSH synthesis. GSH is synthesized by the sequential action of γ-glutamyl-cysteine synthetase (γ-GCS), the rate-limiting enzyme and GSH synthetase. It has been found, that GSH depletion increases the sensitivity of tumour cells to the cytotoxic effects of alkylating compounds and ionizing radiation [53-55]. The thiol antioxidants NAC and GSH have been found by other authors to protect human cancer cells from the toxicity of PTX [38]. GSH level was also associated with resistance of tumour cells towards chemotherapy and depletion of cellular GSH has been found to result in resistance to taxol. Moreover, buthionine sulfoximine which is an inhibitor of glutathione synthesis, was shown to sensitize human breast cancer cells to the toxicity of PTX [56-59].

CONCLUSION

Our results indicate the hepatotoxic effects of doxorubicin and taxanes - paclitaxel and docetaxel, in experimental rat model *in vivo*. Combination of doxorubicin with any of the taxanes enhanced oxidative stress and caused greater changes in investigated parameters of oxidative stress compared to monotherapy treatment. This indicates the development of considerable oxidative stress in liver tissue during treatment with doxorubicin-taxane chemotherapy and possible involvement of oxidative stress in the hepatocyte damage. It cannot be excluded that in the liver, similarly to the heart, taxanes can increase conversion of doxorubicin to the more toxic metabolite - doxorubicinol, which process can contribute to the hepatoxicity of these drugs. However, detailed studies on molecular mechanisms of hepatotoxic effects of combined doxorubicin-taxanes therapy are still missing. Our studies revealed changes in oxidative stress parameters in the rat liver but in other tissues the observed effects can be different. Molecular mechanisms responsible for an increase of hepatotoxicity, cardiotoxicity and neurotoxicity of DOX-taxanes polychemotherapy require further investigation.

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