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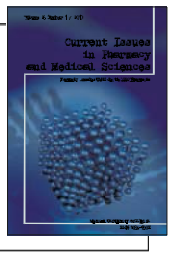

Doxorubicin induces delayed heart and liver mitochondrial depolarisation

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ABSTRACT

The effect of repeated administration of doxorubicin (DOX) on heart and liver morphology, mitochondrial function and redox equilibrium was investigated in rats, contributing in explanation of delayed cardiomyopathy. Male Wistar rats were weekly *intraperitoneally* exposed to doxorubicin (1.5 mg/kg to achieve cumulative dose of 18 mg/kg). To assess persistence changes the heart and liver were studied 3 weeks after last drug administration. Histological examination did not reveal any significant changes in heart and liver in drug-treated animals. However, mitochondrial depolarisation was observed in both organs. These changes in the liver were accompanied by significant increasing in mitochondrial oxidised glutathione and marked decrease of reduced/oxidised glutathione ratio. No changes in cardiac and hepatic NADPH and NADH levels were found. The depolarisation of mitochondria of both studied organs after three weeks since the last injection seems to be a programmed effect of the drug. It may be assumed that this is an early physiological change leading to mitochondrial insufficiency and consequently the cardiac failure.

Keywords: doxorubicin, delayed cardiotoxicity, redox equilibrium, mitochondrial depolarisation

INTRODUCTION

The cardiotoxicity of doxorubicin (DOX) – a broadly used anticancer drug – is related to reactive oxygen species (ROS) overproduction leading to oxidative stress. It was surprising that antioxidative protection observed in animals had no anticardiotoxic effect in patients treated with antracyclines [11]. In addition, it was difficult to explain how oxidative stress found in the presence of DOX my cause cardiomyopathy appearing after years. According to Lebrecht and Walker [9], the drug causes oxidative changes in mitochondrial DNA (mtDNA), which is the basic point to disturbance in mitochondrial respiration. Such imbalance results in mistaken respiration proteins synthesis. Electron transfer changes are a subsequent consequence of it leading to secondary ROS overproduction, even in the absence of DOX. These ROS cause the following mtDNA damages, which are responsible for a defective protein synthesis. Such a circle of events is repeated many times and disturbance of mitochondria function augments with passing of time and eventually responses for delayed cardiomyopathy [2, 9]. It was assumed that at the beginning of these chain reactions induced by DOX, a ROS related depolarisation of the inert mitochondrial membrane must appear. The majority of previous DOX studies, referring to changes in mitochondria membrane potential were conducted in *in vitro* conditions, *ex vivo* in isolated mitochondria of rat heart perfused with DOX or *in vitro* but using over clinical relevant dose of DOX [12, 18]. However, these models are not sufficient to study the mechanism conducted in a delayed cardiomyopathy. For these reasons, the aim of the study was to evaluate mitochondrial inner-membrane depolarisation and markers of redox balance in the heart and liver of rats 3 weeks after completing DOX admi- nistration.

METHODS

The use of animals in the study was approved by the Local Bioethical Commission of Medical University of Lublin. The male Wistar strain rats with the initial body

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weight of 160-195 g were maintained in stable conditions at 22°C with a 12-h light/dark cycle and were given standardized granulated fodder LSM® (Agropol; Motycz, Poland). All the animals were randomly divided into two groups (n=6). The animals were weekly intraperitoneally exposed to DOX hydrochloride (1.5 mg/kg; Ebewe Pharma, Unterach, Austria) for twelve weeks, whereas the control group was injected with physiologic *saline*. The samples were obtained three weeks after the last DOX dose.

The heart ventricles and the liver sections were obtained from the animals, which were anesthetized with sodium pentobarbital (Biowet Puławy, Poland). Organ samples were washed with 20 ml of saline, then placed in liquid nitrogen and stored at -75°C until biochemical analysis or fixed in buffered formaldehyde solution and routinely histologically processed.

Mitochondria were isolated using a commercial kit (Sigma-Aldrich, Saint Luis, USA) according to the manufacturer's manual. The outer membrane integrity was assessed by measure of cytochrome c oxidase activity in the presence and absence of the n-dodecyl β-D-maltosidase using commercial kit (Sigma-Aldrich, Saint Luis, USA). The detection of changes in mitochondrial innermembrane electrochemical potential of heart and liver was conducted using the measurement of cationic, lipophilic dye JC-1 fluorescence (Sigma-Aldrich, Saint Luis, USA) in VICTOR 3 microplate reader (Perkin Elmer, Waltham, USA).

Mitochondrial reduced (mtGSH) and oxidized glutathione (mtGSSG) were evaluated using a commercially available kit (Calbiochem, San Diego, USA). Tissue NADPH and NADH concentrations were determined spectophotometricaly using commercial kits and manufactured method (Bio Vision, Milpitas, USA) using Power Wave Microplate Spectrophotometer (Bio-Tek).

The obtained results were expressed as mean ± SD and statistically analyzed by STATISTICA 5.0 software. The statistical significance of differences between control and other groups was evaluated by U Mann-Whitney test. The value of $P \leq 0.05$ was considered as statistically significant.

RESULTS

DOX did not cause any significant changes in cardiac mitochondrial GSH, GSSG concentration (mtGSH, mtGSSG) and mtGSH/mtGSSG ratio (Fig. 1). However, a significant increase in liver mtGSSG level in drug-exposed group and markedly lower mtGSH/mtGSSG ratio was also observed (Fig. 2). The NADH and NADPH concentration did not change in either organs (Fig. 3 and 4). The inner mitochondrial membrane potential was significantly lower in liver and heart of animals treated with DOX than in the untreated control group (Fig. 5 and 6).

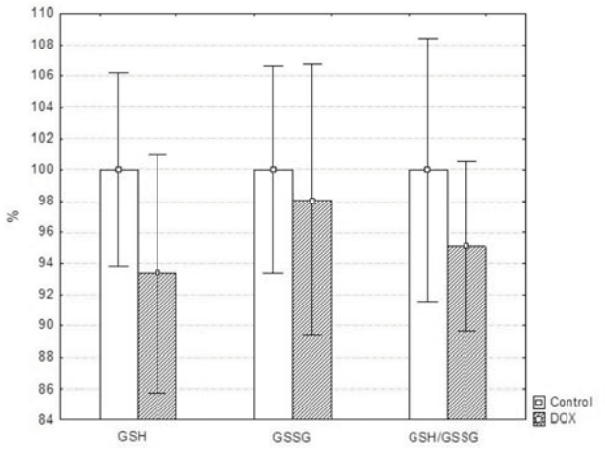

Fig. 1. The content of mtGSH, mtGSSG and mtGSH/mtGSSG ratio (mean ± SD) in cardiac homogenates expressed as percent changes with respect to the control group, which was established at 100%

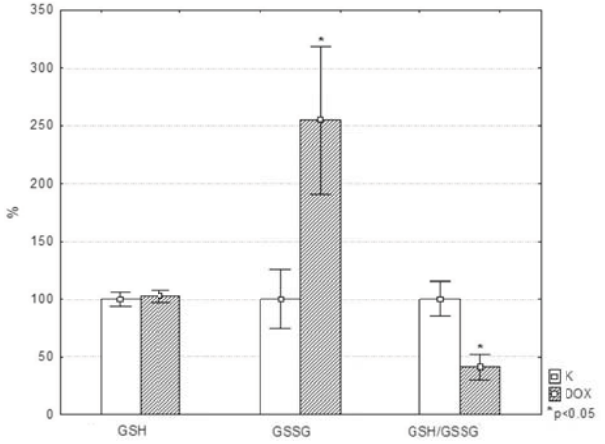

Fig. 2. The content of mtGSH, mtGSSG and mtGSH/mtGSSG ratio (mean ± SD) in liver homogenates expressed as percent changes with respect to the control group, which was established at 100%

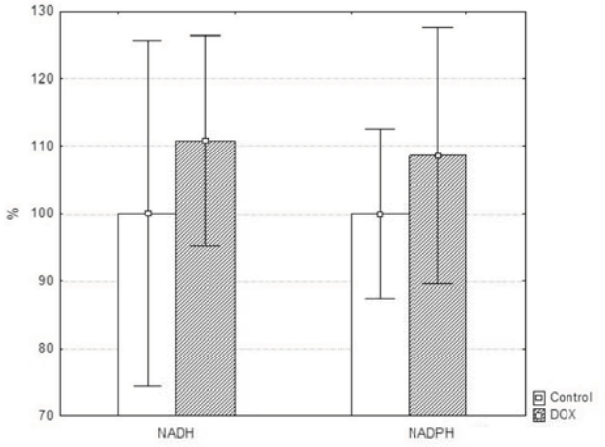

Fig. 3. NADH and NADPH content (mean ± SD) in cardiac homogenates expressed as percent changes with respect to the control group, which was established at 100%

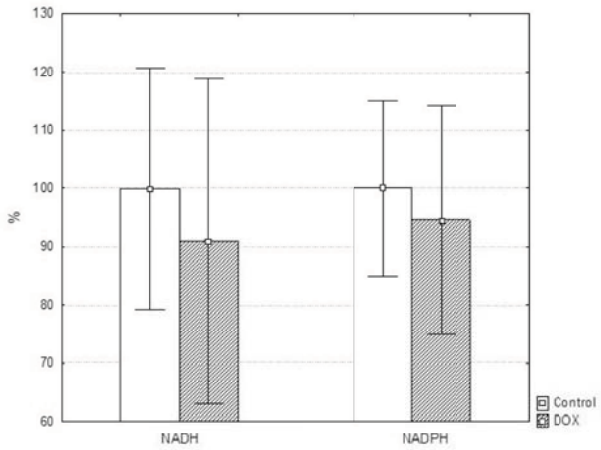

Fig. 4. NADH and NADPH content (mean ± SD) in liver homogenates expressed as percent changes with respect to the control group, which was established at 100%

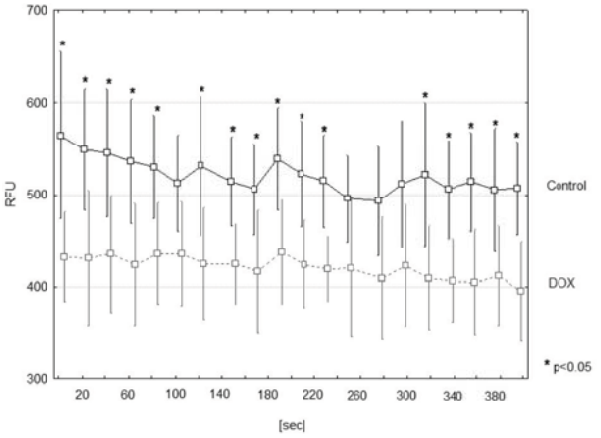

Fig. 5. The intensity of fluorescence of JC-1 dye in isolated cardiac mitochondria (mean ± SD) dependent on inner membrane potential

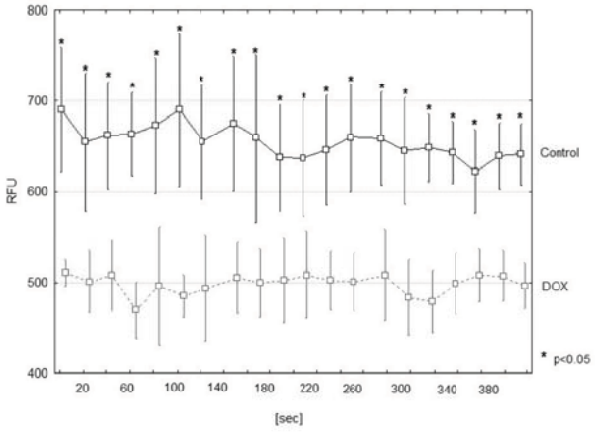

Fig. 6. The intensity of fluorescence of JC-1 dye in isolated liver mitochondria (mean ± SD) dependent on inner membrane potential

DISCUSSION

The current study established that DOX decreased the cardiac and hepatic mitochondrial inner-membrane electrochemical potential, which was accompanied by insignificant changes in mtGSH/mtGSSG ratios and in NADPH and NADH concentrations in homogenates of both organs.

The mitochondrial DNA (mtDNA) is more sensitive to oxidative damages than nuclear DNA (nDNA). Cardiotoxicity mechanism of DOX is multifactorial but in the majority of acute toxicity studies this compound is responsible for oxidative stress in normal cells [11]. In the beginning, anion superoxide radical (O_2^{*-}) production arises when DOX transfers electron from NAD(P)H on O_2. The first step of this process is catalysed by oxidoreductases. O_2^{*-} is transformed to other reactive oxygen species, e.g. H_2O_2, *OH and NOO^- resulting in oxidative stress [4]. The activity of theses oxidoreductases catalysing DOX is much higher in the liver than in the heart [1, 3]. However, in the heart, the activity of antioxidative enzymes is much lower than in the liver [11]. It was also established that the main source of ROS in cardiomyocytes in the presence of DOX is the mitochondrial complex I [1, 11]. When DOX is removed from the tissues, ROS overproduction should be stopped. However, clinically the latent period of cardiac failure reach even years. It may be suspected that DOX programs cardiac cells to be destroyed.

In the current study the organ changes were evaluated three weeks after the last DOX administration (1.5 mg/kg x every 12 weeks; cumulative dose 18 mg). Thus, organ samples were taken at the time when the drug had been excluded from the body [13]. The observed changes in liver and heart mitochondrial membrane potential are thus the effect of the cumulative dose but not a sum of effects of the cumulative and the acute effect of the last dose. The obtained results are consistent with "DOX programmed" thesis by Lebrecht and Walker [9]. It may be assumed that ROS generation in the presence of DOX cause mtDNA damages. The mtDNA ROS related mutation is responsible for disturbance in mitochondrial electron transition leading to secondary ROS overproduction and mtDNA damages. With the passing of time, mitochondria dysfunction reaches the critical point of insufficiency manifesting the cardiac failure resulting from contractility disorders. It seems reasonable that in DOX related oxidative mitochondria disorders the first step is related to a primary DOX induced ROS synthesis and with the passage of time secondary ROS generation dependent on DOX programmed mtDNA mutation appears. Under the conditions of mitochondrial oxidative stress some proteins make a complex of mitochondrial permeability transition (MPT) pore, which allows, in contrast to normal conditions, the passage of molecules over 1.5 kD in size and results in the loss of mitochondrial membrane potential [6, 16]. The MPT was shown to mediate mitochondrial proapoptotic events and initiate the cell death [5]. Spolla-

rosa *et al.* [17] used cultured cells and they showed that DOX-related proapoptotic signals are normalized by an addition of antioxidant thus found participation of oxidative stress in apoptotic events induced by the drug. Furthermore, it was sated that even small damage of cardiomyocytes can lead to cardiomyopathy [20].

Mitochondrial ROS overproduction must be controlled by the antioxidative system, including the most important one related to glutathione. DOX induced cardiotoxicity is critically depended on mitochondrial GSH (mtGSH) [10]. Thus, in the current study, mtGSH and mtGSSG assays were conducted and mtGSH/mtGSSG ratio was calculated. However, the glutathione system was unaffected in cardiac homogenates among drug-exposed rats. It may suggest that in the tested time there was no apparent oxidative stress symptom or the buffeting capacity was sufficient even in ROS overproduction conditions, probably as a result of adaptive processes.

Unlike in the heart, in liver an increase in mtGSSG level and simultaneously decrease in mtGSH/mtGSSG ratio was fund in DOX-treated animals. It seems that mitochondrial redox equilibrium in liver is much stronger affected by antracyclin than in the heart. However, hepatocytes possess incomparable better regenerative properties that cardiomyocytes and in the final results in clinical conditions, the heart not the liver failure is the most common and dangerous life-threatening complication.

The critical molecule in GSH regeneration from GSSG is NADPH. On the other hand, NADPH is a key cofactor for DOX reducing enzymes, *e.g.* iNOS, NADPH cytochrome P450 reductase triggering ROS generation [4]. Thus, NADPH is seemingly paradoxically the molecule promoting oxidative stress and an essential factor in the antioxidative defence system. In the present study, 3 weeks after completing DOX administration there were no changes in the cardiac and hepatic NADPH levels. Taking into account that NADPH is a cofactor of both DOX related oxidative stress and regeneration of antioxidative force (regeneration GSH from GSSG), lack of any significant changes in NADPH level may be explained by the efficiency of NADP rebuilding system. NADPH regeneration is mainly dependent on G6PDH (dehydrogenase glucose 6 phosphate) and the activity of malic enzyme. Moreover, NADPH may be synthesised in the following reaction $NADH+NADP^+ \rightarrow NAD + NADPH$, which is catalysed by $NAD(P)^+$ transhydrogenase [7]. However, insignificant NADPH concentration differences in the heart and liver among rats receiving DOX was accompanied by lack of NADH changes. The obtained data suggests that the four-month study did not elicit stable disturbances in NADPH and NADH levels. In contrast, some previous experiments revealed an inhibiting effect DOX on mitochondrial respiration via the influence on the activity of mitochondrial complexes [19]. In parallel, complex DOX with mitochondrial cardiolipin augmented one electron oxygen reduction to O_2^{*-}. The observed differences probably result from a different schedule of DOX administration. In the current study, three weeks after the last DOX injection it was possible to exclude the primary and direct effect of the drug on redox equilibrium.

It should be pointed out that the permanent changes in inert mitochondria membrane depolarisation was significantly important in the heart and the liver three weeks after administration of the cumulative dose of DOX (18mg/kg). Referring to the heart, the obtained data are consisted with the previous study of Kawasaki *et al.* [8], in which DOX was intraperitoneally administered (2.5 mg/kg 6-time over the period of 2 weeks) and observations were conducted 1 day or 3 and 6 weeks after the last injection. A decrease of ATP and the carnitine level with the progress of time was found. The obtained results are also similar to data published by Sokolove [15], who demonstrates DOX-dependent appearance of MPT pores of the isolated and incubated rat heart mitochondria. The study of cardiac muscular tissue transcripts after 5 weeks since the cumulative dose of 12 mg DOX also showed that mitochondria are the key targets in DOX toxicity [2].

Interestingly, the rate of depolarisation in the heart and the liver was roughly the same despite the fact that the liver concentration of mtGSSG and mtGSH was about 150-fold higher than in the heart. However, the heart and liver mtGSH/mtGSSG ratio was at the same level and in both organs DOX did not cause any changes in this parameter, which is consistent with the study by Pointon *et al.* [14]. The morphology of both organs was similar in control and DOX-exposed groups, which suggests that mitochondrial depolarisation is an earlier event in the cumulative model of DOX cardiotoxicity. Solem et al. [16], who reported a continued mitochondrial dysfunction prior to histopathological significant changes, presented similar data.

The current and previously published results considered that depolarisation of the heart and liver mitochondria after three weeks since the last DOX injection seems to be a programmed effect of the drug. It may be assumed that this is an early physiological change leading to mitochondrial insufficiency and consequently the cardiac failure. The mitochondrial redox equilibrium is affected by the drug much stronger in liver than in heart. The future studies should focus on changes on inert mitochondrial membrane depolarisation and mitochondria redox changes. A longer time intervals since completing DOX administration will be also desirable. Such a comparative assessment of mitochondrial changes in the heart and the liver could explain why despite staring similarities, cardiotoxicity, but not hepatotoxicity, is the major complication of DOX therapy.

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