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Prevention of Doxorubicin-induced cardiomyopathy by reduced glutathione

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Summary. The aim of the present investigation was to evaluate the potential cardioprotective effect of reduced glutathione (GSH) against the delayed cardiomyopathy induced by doxorubicin (DXR) in a well-documented rat model. DXR was administered i.v. at a weekly dose of 3 mg/kg for a total of 4 doses; 250 or 500 mg/kg of GSH was given i.v. 10 min before and 2 h after each DXR injection, resulting in a total weekly dose of 500 or 1000 mg/kg, respectively. The development of cardiotoxicity was monitored in vivo by means of electrocardiography (QaT duration), and was evaluated by measuring the contractile performance of isolated atria and by light and electron microscopy of left ventricular samples excised 5 weeks after the last DXR administration. DXR was found to impair body weight gain and to produce an irreversible and time-dependent prolongation of QaT, a decrease in myocardial contractility of isolated atria and typical morphologic alterations, including myocyte vacuolization and myofibrillar loss. Pretreatment with GSH at a dose of 500 mg/kg × 2, but not at 250 mg/kg × 2, partially prevented the impairment of body weight gain, QaT prolongation in ECG and the decrease in myocardial contractility of isolated atria induced by DXR. Alterations of the morphologic pattern were also significantly reduced in animals receiving the higher dose of GSH. Determinations of the cardiac non-protein sulfhydryl group content showed that GSH, at doses higher than or equal to 500 mg/kg, significantly increased this parameter, irrespective of the presence of DXR. In conclusion, the present data indirectly support the hypothesis that oxidative damage is involved in DXR cardiotoxicity and indicate that maintenance of the reduced thiol pool could be an important issue in myocardial protection.

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Introduction

The clinical use of doxorubicin (DXR) in long-term treatments is limited by serious side-effects and particularly by the development of a dose-dependent form of cardiomyopathy, which is frequently lethal [26, 28]. Several reports suggest that oxygen-free radicals produced during the metabolic activation of DXR may have toxic effects on heart muscle [4, 15], which is provided with poor mechanisms of detoxification of such species [8]. In some animal models DXR has been shown to reduce non-protein sulfhydryl group levels in myocardial cells [7, 15], and the administration of exogenous reduced glutathione (GSH) was found to prevent the depletion of the reduced thiol pool after DXR treatment [7], as well as the development of acute DXR cardiotoxic effects in mice, without affecting the antitumor properties of the drug [27]. A similar protective effect was observed in animals treated with DXR and *N*-acetylcysteine, a putative precursor of GSH [9]. The aim of the present investigations was to evaluate whether the administration of GSH protected the myocardium against the delayed toxicity of DXR in a validated model of cardiotoxicity in rats [22]. The possibility of a relationship between the hypothesized protective effect of GSH and the myocardial levels of GSH after i.v. administration of this compound was also investigated.

Materials and methods

Animals. Female CD (Crl:CD(SD)BR) rats (Charles River, Calco, Italy) with a starting weight of 120–130 g were used in the study. The animals were maintained under standard laboratory conditions and in quarantine for 7 days before being randomized into experimental groups of 6–8 animals.

Drugs and treatment schedules. DXR and reduced GSH were kindly supplied by Farmitalia-C. Erba (Milan, Italy) and by Boehringer Biochemia Robin, (Milan, Italy), respectively. DXR was dissolved in distilled water and administered i.v. at a dose of 3.0 mg/kg per week (2 ml/kg) for 4 weeks. Reduced GSH was reconstituted in distilled water immediately before use and was given i.v. at a dose of 250 or 500 mg/kg

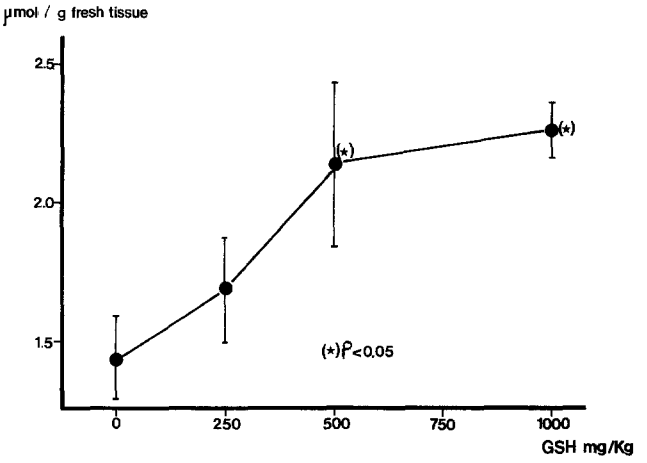

Fig. 1. Effect of different doses of GSH on the non-protein sulfhydryl content in the heart (means ± SEM of six determinations) performed 30 min after GSH administration

(2 ml/kg) 10 min before and 2 h after each DXR administration, resulting in a total weekly dose of 500 or 1000 mg/kg, respectively. Controls received the same volumes of saline as the treated animals (2 ml/kg per injection) according to the same time schedules.

Determinations of non-protein sulfhydryl levels. Groups of 6 animals were sacrificed at different times after a single i. v. administration of different doses of GSH. The hearts were removed, weighed and immediately homogenized in ice-cold 5% trichloroacetic acid; the protein precipitate was removed by centrifugation at 5000 *g* for 10 min and the acid-soluble sulfhydryl concentration was determined by the method described by Ellman [10]. The results of these determinations were used to choose the GSH doses for the subsequent investigations. Non-protein sulfhydryl levels were also determined in hearts from animals receiving 3 mg/kg i. v. of DXR preceded (10 min before) by an i. v. injection of saline or GSH at the doses chosen for the cardiotoxicity study (250 or 500 mg/kg). This set of determinations was performed 30 min after DXR administration. Control values refer to hearts from animals receiving i. v. injections of saline according to the same schedules as the treated animals.

Evaluation of general toxicity. The body weight of the animals was recorded at 2-week intervals, as an index of general toxicity. Five weeks after the last DXR administration, after recording the last ECG, the animals were sacrificed by cervical dislocation. Blood samples for biochemical and hematological analyses were obtained after the sacrifice. A complete blood count, serum determinations of urea nitrogen, creatinine, glucose, sodium, potassium, calcium, iron, lactate dehydrogenase, alkaline phosphatase and transaminases were performed.

Evaluation of delayed cardiotoxicity. The development of myocardial toxicity was monitored in vivo by ECG and was subsequently evaluated by measuring the contractile performance of isolated atria and by light and electron microscopic examination of left ventricular samples excised 5 weeks after the last DXR administration.

1. *ECG parameters.* Electrocardiograms were recorded at the beginning of the treatment and subsequently at 2-week intervals. The standard procedure for ECG recording has been described in detail elsewhere [22]. QaT duration was measured for each tracing since the parameter was found to be related to the severity of the morphologic lesions developed by DXR-treated animals [22, 23]. QaT duration was not corrected for changes in heart rate, according to the findings of some authors [5, 14]. QaT was calculated as the interval between the Q wave and the apex of the T wave.
2. *Assessment of myocardial contractility.* Myocardial contractility was evaluated in spontaneously beating atria isolated from animals at the end of the observation period and incubated in Tyrode's solution at 37° C, aerated with a 95% O_2 + 5% CO_2 mixture, to maintain a pH value of 7.4. The contractile performance of isolated atria was assessed by subjecting the preparations to stepwise increases in resting tension and recorded by means of an isometric tension recording system; dF/dt (g/s) was used as a contractility index.
3. *Histological evaluation.* Immediately after the sacrifice, left ventricular fragments were prepared for electron microscopy, as described elsewhere [21, 22]. DXR cardiotoxicity was evaluated by examination of transverse and longitudinal sections of the whole ventricle and the severity of the lesions was quantitated according to a scale from 0 to 2, as follows: 0 = no damage; 0.2 = involvement of very few (fewer than 10) myocytes; 0.5 = involvement of more than 10 myocytes; 1 = involvement of small clusters of myocardial cells; 1.5 = multiple clusters of vacuolated myocytes; 2 = diffuse myocyte damage. The sections were evaluated without prior knowledge of the treatment given to the animals.

Table 1. Non-protein sulfhydryl group concentration in rat myocardial tissue (µmol/g fresh tissue) 30 min after a single i. v. administration of GSH and/or DXR (means ± SEM of 4–6 determinations)

Controls	1.43±0.16
DXR	1.34±0.13
GSH (250 mg/kg)	1.70±0.24
GSH (500 mg/kg)	2.14±0.31[a, b]
GSH (250 mg/kg) + DXR	1.59±0.19
GSH (500 mg/kg) + DXR	2.08±0.24[a]

$P < 0.05$, [a] vs DXR; [b] vs controls

Statistical analysis. A factorial design of the analysis of variance was adopted to assess differences in the time-courses of body weight and QaT duration and in the contractile responses of isolated atria to increasing values of resting tension. Morphologic data were evaluated by means of Wilcoxon's non-parametric test. Differences in cardiac non-protein sulfhydryl content were evaluated by means of a one-way analysis of variance with Duncan's multiple range test (confidence level $P = 0.05$).

Results

Determination of non-protein sulfhydryl levels

The administration of GSH produced a dose-dependent increase in myocardial sulfhydryl groups, with a peak value observed 10 min and a subsequent plateau up to 30 min. Figure 1 reports the mean peak values of the acid-soluble sulfhydryl content of the heart after a single i. v. administration of different doses of GSH, and shows that only doses equal to or greater than 500 mg/kg were able to increase significantly the reduced thiol pool of myocardial cells. DXR alone did not affect the extent of the myocardial non-protein sulfhydryl pool 30 min after drug administration. When GSH was administered in combination with DXR, a significant increase in non-protein sulfhydryl group levels was observed with the dose of 500 mg/kg, wherease 250 mg/kg were ineffective on this as well as on the other parameters tested in the study (Table 1).

Evaluation of general toxicity

No deaths were observed in any of the experimental groups during the observation period. As shown in Fig. 2, DXR was found to impair the body weight gain significantly

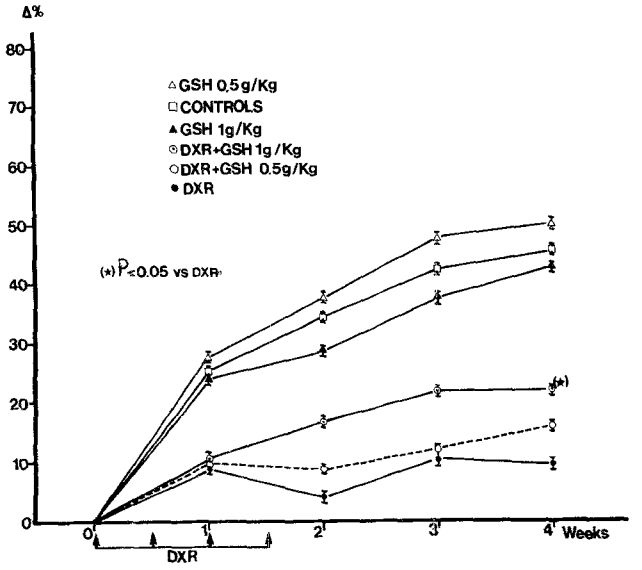

Fig. 2. Time-course of body weight in rats treated with DXR and DXR+GSH at two different doses (means ± SEM of six to eight animals)

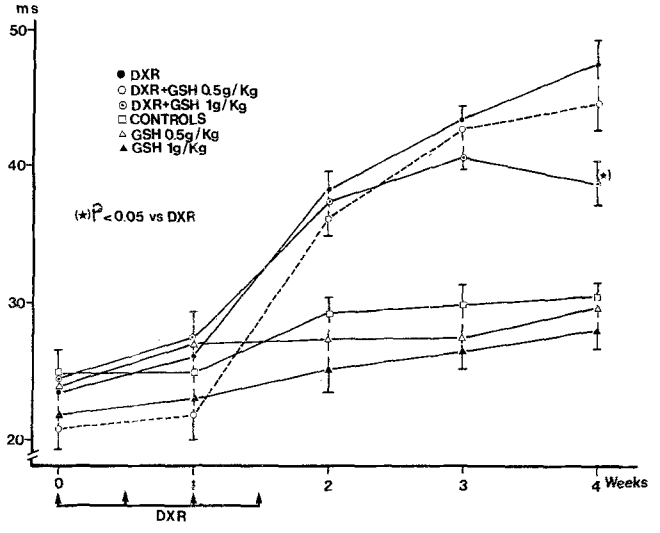

Fig. 3. Time-course of QaT duration in rats treated with DXR and DXR+GSH at two different doses (means ± SEM of six to eight animals)

compared with controls. At the dose of 500 mg/kg × 2 GSH partially prevented the effect induced by DXR, whereas the lower dose was ineffective. No substantial differences were found in serum biochemical and hematological parameters. WBC counts, RBC counts and hemoglobin concentration were not modified by any of the treatments. A slight increase was observed in serum potassium and calcium levels in DXR-treated animals, but the difference from controls was not statistically significant (data not shown).

Evaluation of delayed myocardial toxicity

1. *ECG alterations.* As already observed in previous studies, QaT duration was significantly increased in DXR-

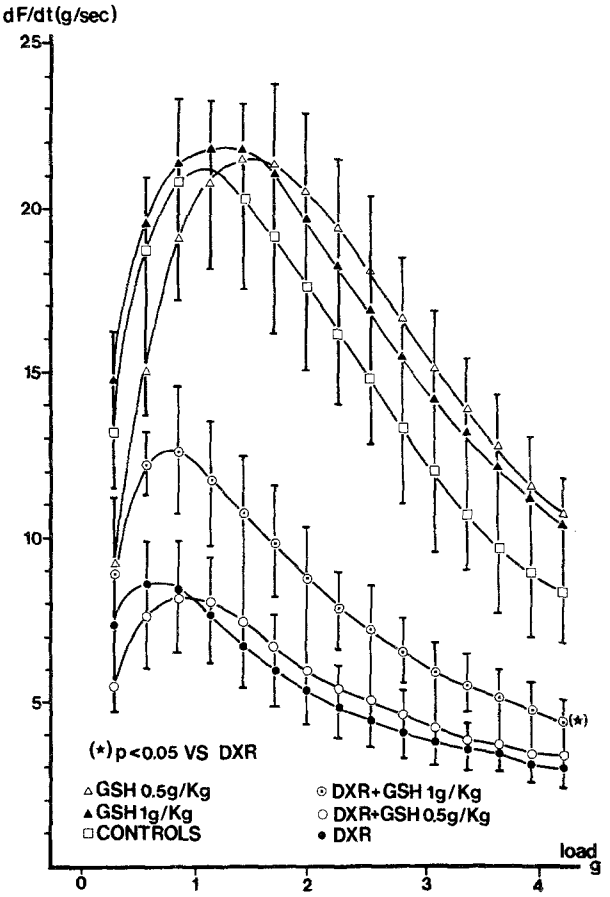

Fig. 4. Contractile response to increasing load of isolated atria excised from rats treated with DXR and DXR+GSH at two different doses (means ± SEM of six to eight animals)

treated animals, particularly from the 4th week onwards. GSH alone did not affect QaT duration, but at the dose of 500 mg/kg × 2 significantly reduced the effect of DXR on this parameter. Again, the lower dose of GSH was devoid of protective effects (Fig. 3).

2. *Assessment of myocardial contractility.* The evaluation of the contractile properties of spontaneously beating atria isolated five weeks after the last DXR administration showed that DXR impaired the contractile performance of preparations isolated from treated animals as against control preparations. Neither dose of GSH alone was found to affect the contractile performance. However, the combined administration of DXR and the higher GSH dose significantly reduced the inhibitory effect exerted by the anthracycline antibiotic (Fig. 4).

3. *Histologic evaluation.* The most prominent findings in left ventricular preparations obtained from DXR-treated animals were represented by myocyte vacuolization and myofibrillar loss; mitochondria were not significantly affected. The damaged cells were randomly distributed throughout the myocardium and no preferential location was observed. Figure 5 reports the mean morphological scores attributed to the different groups of preparations: a slight, non-significant improvement of the morphological pattern was observed in preparations receiving DXR plus the lower GSH dose, but a significant difference was only

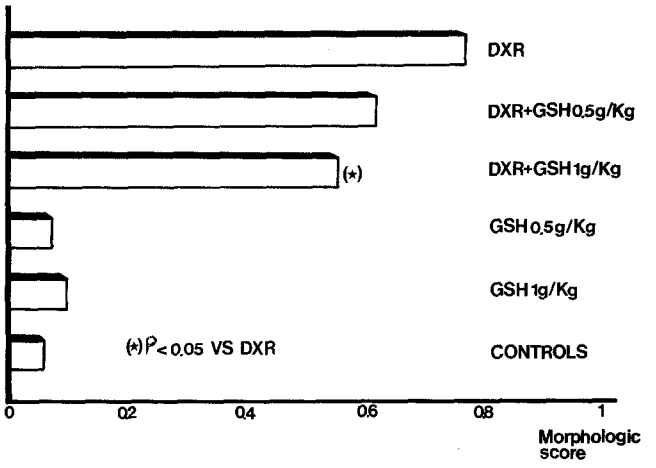

Fig. 5. Morphological evaluation of the effect of two different doses of GSH on the cardiac toxicity of DXR (means of six to eight animals)

observed when GSH was administered at the dose of 500 mg/kg × 2. The incidence of severe morphological lesions (scores greater than or equal to 0.5) was also evaluated: the values obtained (53% for DXR alone, 50% for DXR+GSH 250 mg/kg × 2 and 38% for DXR+GSH 500 mg/kg × 2) confirmed that GSH was able to achieve a dose-dependent degree of protection against DXR-induced myocardial injury.

Discussion

The schedule adopted in the present study for DXR administration in the rat produced general toxic signs, evidenced by a significant impairment of body weight gain, and delayed manifestations of cardiax toxicity, including irreversible prolongation of QaT intervals, impairment of contractility of isolated myocardial preparations and a typical pattern of morphologic alterations which is similar to the pattern observed in patients treated with anthracyclines [11]. The choice of the doses of GSH was based on mortality data reported by other authors, showing that 2000 mg/kg of GSH i.p. produced lethal effects in mice [27], and on the preliminary results reported in the present study, indicating that doses of 500–1000 mg/kg were able to significantly increase the myocardial non-protein sulfhydryl group content. Since in isolated rat hearts free radical formation by DXR has been shown to peak after 10 min of perfusion [19], the schedule of GSH administration was chosen so that high levels of non-protein sulfhydryl groups were achieved during the early stages of the presence of DXR in the myocardium.

With this experimental model, the higher dose of GSH (500 mg/kg × 2) was found to increase the myocardial reduced thiol pool and to reduce the general and cardiac toxicity of DXR, whereas the lower dose (250 mg/kg × 2) was devoid of significant protective effects. In the present investigations, the pharmacokinetics of the GSH-DXR interactions were not directly investigated. However, data obtained in a previous study showed that GSH is able to prevent the cardiotoxic effects of DXR even when the two agents were tested on isolated myocardial preparations [25]. Therefore, it is unlikely that general kinetic factors play a major role in the cardioprotective effects of GSH in vivo. As far as we know, no data are currently available as to the possible interference of GSH with the cellular pharmacokinetics of DXR. However, the pretreatment with GSH is not expected to affect the tissue distribution of DXR on the basis of the following observations.

1. Nucleophilic thiols are unable to react at plasma level with DXR, since the quinone itself is not reactive; an interaction of GSH with reactive intermediates of the drug following intracellular metabolic activation (i.e. redox cycling) has been proposed in organs and tissues, including heart [16].

2. The plasma half-life of GSH is very short (i.e. a few minutes), since it is rapidly removed following i.v. administration [1, 13].

3. Pretreatment of tumor-bearing mice with GSH (at the same dose levels used in the present study) did not interfere with the antitumor activity of doxorubicin (data not shown). This observation is consistent with previous observations that sulfhydryl-containing compounds were not able to change DXR uptake by tumor cells [12].

The partial prevention of DXR-induced cardiotoxicity by GSH seems to support the hypothesis of the oxidative damage in this pathology. In fact, the metabolic activation of DXR to the corresponding semiquinone free radical has been proposed to initiate a reaction cascade, leading to the generation of oxygen-derived reactive species, such as superoxide anions, hydrogen peroxide and hydroxyl radicals [3, 4, 6, 21], which in turn would result in extensive membrane lipid peroxidation and DNA damage [15, 17]. The production of an excess of oxygen metabolites is particularly critical in the heart, which is provided with limited enzymatic defenses against these species [8]. Three different enzyme systems are involved in the removal of oxygen metabolites from the cell: superoxide dismutase (SOD), catalase and selenium-dependent glutathione peroxidase (GSH-Px). SOD and catalase activities were found to be significantly lower in the myocardium than in the liver [8], which is also known to activate DXR, but is not a target for the organ-specific toxicity of the drug. It seems therefore that the ability to dispose efficiently of oxygen metabolites plays an important role in protecting tissues against DXR toxicity and that the heart mainly relies in GSH-Px to perform this task. However, DXR has been shown to depress the activity of this enzyme in the heart [8, 20] and to reduce the myocardial pool of GSH [7], thus increasing the intracellular concentration of oxygen-derived toxic metabolites by both promoting their generation and inhibiting their enzymatic removal. The use of antioxidants to limit the extent of the myocardial damage produced by oxygen-free radicals has been extensively emphasized in the recent literature [18]. Since sulfhydryl groups play an important role in promoting the nonenzymatic detoxification of free radicals [2], it has been hypothesized that increasing the cardiac sulfhydryl group content might enhance the ability of the heart to withstand DXR exposure; the results obtained in DXR-treated mice receiving *N*-acetylcysteine seem to support this hypothesis [9]. The partial protection observed in this study when DXR-treated rats received ade-

quate doses of GSH also fits into this pattern. In fact, the degree of cardioprotection achieved with the different doses of GSH seems to be related to their ability to increase the sulfhydryl group content during the time of persistence of DXR in myocardial cells, although DXR did not significantly deplete the non-protein sulfhydryl pool in this rat model (but see [27] for similar results in the mouse).

The cardioprotective effect of GSH suggests that the delayed as well as the acute manifestations of DXR cardiotoxicity may depend on the early oxidative damage produced by the drug and that maintenance of an adequate reduced thiol pool may represent an important approach towards myocardial protection.

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