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ORIGINAL ARTICLE

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The influence of the time interval between monoHER and doxorubicin administration on the protection against doxorubicin-induced cardiotoxicity in mice

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Abstract *Purpose*: Despite its well-known cardiotoxicity, the anthracyclin doxorubicin (DOX) continues to be an effective and widely used chemotherapeutic agent. DOX-induced cardiac damage presumably results from the formation of free radicals by DOX. Reactive oxygen species particularly affect the cardiac myocytes because these cells seem to have a relatively poor antioxidant defense system. The semisynthetic flavonoid monohydroxyethylrutoside (monoHER) showed cardioprotection against DOX-induced cardiotoxicity through its radical scavenging and iron chelating properties. Because of the relatively short final half-life of monoHER (about 30 min), it is expected that the time interval between monoHER and DOX might be of influence on the cardioprotective effect of monoHER. Therefore, the aim of the present study was to investigate this possible effect. *Methods*: Six groups of 6 BALB/c mice were treated with saline, DOX alone or DOX (4 mg/kg i.v.) preceded by monoHER (500 mg/kg i.p.) with an interval of 10, 30, 60 or 120 min. After a 6-week treatment period and additional observation for 2 weeks, the mice were sacrificed. Their cardiac tissues were processed for light microscopy, after which cardiomyocyte damage was evaluated according to Billingham (in Cancer Treat Rep 62(6):865–872, 1978). Microscopic evaluation revealed that treatment with DOX alone induced significant cardiac damage in comparison to the saline control group ($P < 0.001$). *Results*: The number of damaged cardiomyocytes was 9.6-fold (95% CI 4.4–21.0) higher in mice treated with DOX alone than that in animals of the control group. The ratio of aberrant cardiomyocytes in mice treated with DOX preceded by monoHER and those in mice treated with saline ranged from 1.6 to 2.8 (mean 2.2, 95% CI 1.2–4.1, $P = 0.019$). The mean protective effect by adding monoHER before DOX led to a significant 4.4-fold reduction ($P < 0.001$, 95% CI 2.3–8.2) of abnormal cardiomyocytes. This protective effect did not depend on the time interval between monoHER and DOX administration ($P = 0.345$). *Conclusion*: The results indicate that in an outpatient clinical setting monoHER may be administered shortly before DOX.

Keywords MonoHER · Monohydroxyethylrutoside · Doxorubicin · Cardiotoxicity · Protection

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Introduction

Doxorubicin (DOX) is a potent antitumor agent and is widely used to treat a range of cancers, including solid tumors and lymphomas. A major limitation for its use is the development of cardiomyopathy at high cumulative doses (> 550 mg/m^2) [1, 2, 3]. Although several hypotheses concerning the cause of DOX-induced cardiotoxicity have been suggested in the literature, the

formation of free radicals by DOX semiquinones is supposed to be the most important mechanism of DOX-induced cardiotoxicity [4, 5]. The cardiac myocyte is particularly susceptible to the free radicals because protective enzymes, like superoxide dismutase (SOD), catalase and GSH-peroxidase are present in a lower concentration in heart tissue than in to other tissues [1, 6]. The generation of free radicals is facilitated by the formation of DOX–iron complexes [7, 8]. Scavengers of free radicals and iron chelators showed a protective effect against DOX- induced free radical production and cardiotoxicity in vitro and in vivo [9, 10, 11]. Up to now, the cardioprotectant dexrazoxane, a chelating agent that binds iron intracellularly, is the only drug that has been cautiously introduced into the clinic in patients treated with anthracyclines [12].

The histopathological features of DOX-induced cardiac damage, characterized by endomyocardial biopsies of patients and laboratory animals, include the loss of myofibrils and vacuolar degeneration [13, 14].

In previous studies we have shown the iron chelating and radical scavenging properties of monohydroxyethylrutoside (monoHER) [15, 16]. Because of these properties, monoHER was tested as a protectant against DOX-induced cardiotoxicity. It appeared that monoHER protected the heart tissue of mice against DOX-induced cardiotoxicity in vivo [17] and ex vivo [18]. In vitro and in vivo experiments also showed that monoHER did not interfere with the antitumor effect of DOX [19].

The cardiotoxicity of DOX is not only related to its cumulative dose but also to its peak plasma drug concentration [20, 21]. Considering this, it seems plausible that maximal monoHER concentrations should be present during the highest circulating concentrations of DOX, assuming that a maximal plasma concentration of monoHER offers a maximal protection against DOX-induced cardiotoxicity. Because of the relatively short final half-life of monoHER (about 30 min), it is expected that the time interval between monoHER and DOX might be of influence on the cardioprotective effect of monoHER. Therefore, the aim of the present investigation was to evaluate the influence of the time interval between monoHER and DOX administration on the protection against DOX-induced cardiotoxicity by evaluating the morphological changes in the cardiac tissue of mice treated with different time intervals between monoHER and DOX administration.

Materials and methods

Chemicals

7-Monohydroxyethylrutoside (monoHER) was kindly provided by Novartis Consumer Health (Nyon, Switzerland). The drug was formulated under aseptic conditions by the Pharmacy Department, VU medical center, Amsterdam, The Netherlands. The required amount of monoHER was dissolved in 20 ml dextrose 5% for intraperitoneal (i.p.) use, adjusted to pH = 9.3 using sodium hydroxide 4 M. After dissolution of the drug, the solution was readjusted to pH = 8.4 with hydrochloric acid 1 M, giving a final concentration of 33 mg/ml. Formulated DOX (Doxorubicin hydrochloride, 2 mg/ml) was obtained from Pharmachemie B.V. (Haarlem, The Netherlands). Before injection, the content of the vial was dissolved in sterile 0.9% NaCl solution to a concentration of 1 mg/ml.

Animals

Thirty-six male BALB/c mice (20–25 g) obtained from Harlan Nederland (Horst, The Netherlands) were kept in a light- and temperature-controlled room (21–22°C; humidity 60–65%). The animals were fed a standard diet (Hope Farms, Woerden, The Netherlands) and allowed to eat and drink tap water ad libitum. The animals were allowed to adapt to the laboratory housing conditions for 2 weeks before starting the experiment.

Experimental design

The protocol was approved by the ethics committee for animal experiments of the Vrije Universiteit (Amsterdam, The Netherlands).

With an interval of 2 months, two groups of 18 mice (each subdivided into six groups of 3 mice) were treated according to one of the following schemes for 6 weeks:

Group 1 ($n=6$)	0.5 ml 0.9% NaCl solution i.p., followed by 0.1 ml 0.9% NaCl solution i.v.
Group 2 ($n=6$)	0.5 ml 0.9% NaCl solution i.p., followed by 4 mg/kg DOX i.v.
Group 3 ($n=6$)	500 mg/kg monoHER i.p., followed by 4 mg/kg DOX i.v. after 10 min.
Group 4 ($n=6$)	500 mg/kg monoHER i.p., followed by 4 mg/kg DOX i.v. after 30 min.
Group 5 ($n=6$)	500 mg/kg monoHER i.p., followed by 4 mg/kg DOX i.v. after 60 min.
Group 6 ($n=6$)	500 mg/kg monoHER i.p., followed by 4 mg/kg DOX i.v. after 120 min.

The i.v. injections were administered in the tail vein. During treatment and 2 weeks of observation thereafter, body weight was determined once a week as a measure of general toxicity. After the 8-week period, the animals were sacrificed. The hearts were excised and the central parts of both ventricles were cut into 5-mm-thick pieces of 2–3 mm, which were fixed in 2% phosphate buffered glutaraldehyde solution.

Histological analyses

After fixation in 2% phosphate buffered glutaraldehyde solution, the heart tissues were postfixed in 1% osmium tetroxide. The tissues were then dehydrated through a

graded series of ethanol solutions of 70–95% and embedded in JB-4 Plus resin. Thereafter, 0.5–3.0 μm-thick sections were cut with a glass knife. These semithin sections were examined by light microscopy and the myocardial damage was evaluated according to Billingham [13]. For this purpose, the percentage of cardiac myocytes, which had been damaged, was established. Cardiac myocytes with more than two vacuoles and/or loss of myofibrils were considered to be damaged. The circumference of the scoring area was measured using a commercially available interactive video overlay based measuring system (Q-Prodit, Leica, Cambridge, UK) [22]. For each mouse, the number of aberrant myocytes/mm^2 was scored.

Statistical analysis

For the analyses, the number of aberrant cardiac myocytes was log-transformed yielding normally distributed variables. Differences between experimental groups were assessed using two-sided t tests. The influence of time between monoHER and DOX administration on the number of aberrant myocytes was examined by linear regression. The level of significance chosen was 5% ($P < 0.05$). All calculations were done with SPSS version 9.0.

Results

Behavior of the animals appeared normal in all treatment groups. Animals appeared lively throughout the study and there were no signs of decreased activity, which would indicate low general toxicity. No significant differences in weight gain between the groups were observed.

Microscopic evaluation revealed that treatment with DOX alone induced significant myocardial damage in comparison to the saline-treated control group, which was demonstrated by the appearance of vacuoles ($P < .001$). Treatment with DOX alone showed a 9.6-fold (95% CI 4.4–21.0) increase of damaged cardiac myocytes compared to the control group. Loss of myofibrils was not found.

The mean ratio of aberrant cardiac myocytes/mm^2 in treated versus the saline control group is presented in Table 1. When monoHER was added before DOX treatment, the mean presence of aberrant cardiac myocytes was 2.2-fold (95% CI 1.2–4.1) higher than that in the saline control group ($P = 0.019$). Compared to the mice treated with DOX alone, the addition of monoHER before DOX showed a significant ($P < 0.001$) protective effect by reducing the mean ratio of aberrant myocytes with a factor 4.4 (95% CI 2.3–8.2). The protective effect did not depend on the time interval between monoHER and DOX administration ($P = 0.345$).

Table 1 Ratio of aberrant cardiac myocytes/mm^2 in treated versus control (=saline treated) mice (95% CI)

Group ($N = 6$ per group)	Ratio (95% CI)
1. Saline	Reference
2. DOX	9.6 (4.4–21.0)
3. MH 10 min before DOX	1.9 (1.1–3.5)
4. MH 30 min before DOX	1.6 (0.8–3.1)
5. MH 60 min before DOX	2.8 (1.6–5.1)
6. MH 120 min before DOX	2.5 (1.3–4.8)

Discussion

In the past it was shown that the presently used DOX schedule, i.e., 4 mg/kg/DOX once every week for 6 weeks, results in an appreciable cardiotoxicity which is well suited to investigate cardioprotectors and still acceptable for the animal [23]. It had also been demonstrated that the flavonoid monoHER is a very effective protector against DOX-induced cardiotoxicity in vitro and in vivo without interfering with the antitumor effect of DOX [24, 17, 19]. It also appeared that monoHER injected in mice as an i.p. dose of 500 mg/kg did not exert any effect on their heart tissue [25].

Van Acker et al. [17] showed the protection of monoHER against DOX-induced cardiac damage in mice when given once as an i.p. dose of 500 mg/kg 1 h before DOX. After an i.v. bolus injection of DOX, maximal plasma levels are immediately obtained and also various tissues, including the heart, immediately achieve high concentrations of DOX (followed by a further distribution and a slow elimination phase) [26]. High peak serum levels, which follow after rapid administration, seem to be correlated with cardiotoxicity. Legha et al. [27] showed that decreasing peak plasma levels of DOX by using continuous infusion reduced cardiotoxicity. Also DOX divided over a weekly schedule is associated with less anthracycline-induced cardiac damage than the same amount of DOX given in the conventional 3-week schedules [28]. Because of these considerations, high concentrations of monoHER should be present during the peak concentrations of DOX. Pharmacokinetic (pk) studies of i.p. administered monoHER in mice showed that C_{max} was obtained at about 10 min after administration in plasma as well as in tissues. Thereafter, monoHER disappeared rapidly within a few hours [29]. Based on these findings and the mechanism of action of monoHER—i.e., scavenging of DOX-induced radicals—it was postulated that protection by monoHER could be optimized by giving DOX 10 min after i.p. monoHER. Our data, however, do not indicate a significantly better protection against DOX-induced cardiac damage when reducing the time interval between monoHER and DOX from 2 h to 10 min.

In this experiment, and also in earlier studies, the protective properties of monoHER were evaluated after an additional observation time of only 2 weeks after the 6-week treatment period. Several clinical studies showed

that the incidence of ventricular dysfunction continues to increase with time [30, 31, 32]. It is therefore important to know whether the protective effect of monoHER is still present after a longer period of time and it may be that the influence of the time interval between monoHER and DOX administration becomes of significant importance during such a longer observation period. The present data, however, suggest that in a (out-patient) clinical setting monoHER may be administered in a convenient short time before DOX.

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