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Paradoxically, iron overload does not potentiate doxorubicin-induced cardiotoxicity *in vitro* in cardiomyocytes and *in vivo* in mice

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ABSTRACT

Doxorubicin (DOX) is known to induce serious cardiotoxicity, which is believed to be mediated by oxidative stress and complex interactions with iron. However, the relationship between iron and DOX-induced cardiotoxicity remains controversial and the role of iron chelation therapy to prevent cardiotoxicity is called into question.

Firstly, we evaluated *in vitro* the effects of DOX in combination with dextran–iron on cell viability in cultured H9c2 cardiomyocytes and EMT-6 cancer cells. Secondly, we used an *in vivo* murine model of iron overloading (IO) in which male C57BL/6 mice received a daily intra-peritoneal injection of dextran–iron (15 mg/kg) for 3 weeks (D0–D20) and then (D21) a single sub-lethal intra-peritoneal injection of 6 mg/kg of DOX.

While DOX significantly decreased cell viability in EMT-6 and H9c2, pretreatment with dextran–iron (125–1000 μg/mL) in combination with DOX, paradoxically limited cytotoxicity in H9c2 and increased it in EMT-6. In mice, IO alone resulted in cardiac hypertrophy (+22%) and up-regulation of brain natriuretic peptide and β-myosin heavy-chain (β-MHC) expression, as well as an increase in cardiac nitro-oxidative stress revealed by electron spin resonance spectroscopy. In DOX-treated mice, there was a significant decrease in left-ventricular ejection fraction (LVEF) and an up-regulation of cardiac β-MHC and atrial natriuretic peptide (ANP) expression. However, prior IO did not exacerbate the DOX-induced fall in LVEF and there was no increase in ANP expression. IO did not impair the capacity of DOX to decrease cancer cell viability and could even prevent some aspects of DOX cardiotoxicity in cardiomyocytes and in mice.

Introduction

Doxorubicin (DOX), an anthracycline antibiotic, is a broad-spectrum anticancer drug, particularly useful in the treatment of malignant lymphomas, acute leukemia, sarcomas and solid tumors such as breast, lung and ovary cancers (Young et al., 1981). However, despite good therapeutic results, the clinical use of DOX in chemotherapy is limited because it causes acute, sub-acute, and chronic cardiotoxicity (Horan et al., 2006; Torti et al., 1986) leading to dose-dependent congestive heart failure, which may occur several years after treatment cessation (Steinherz et al., 1991), and may affect up to 20% of patients (Chatterjee et al., 2010; Singal and Iliskovic, 1998).

Several putative molecular mechanisms have been proposed to explain the cardiotoxicity of anthracyclines, but while the exact mechanism is still a matter of debate, it seems to be distinct from its anti-tumor activity since cardiomyocytes are minimally replicating cells (Shi et al., 2011). Oxidative stress is believed to be an important pathway in the cardiac side-effects of anthracycline therapy (Delemasure et al., 2007; Ghibu et al., 2012; Richard et al., 2011, 2008) and is related to the production of reactive oxygen and nitrogen species (RONS) during the intracellular metabolism of the quinone (Myers et al., 1977). Additionally, DOX forms stable complexes with ferric iron (Myers et al., 1982), and the iron in the complex undergoes reduction to ferrous iron, resulting in the generation of a semiquinone free radical of DOX (Gutteridge, 1984). The semiquinone then reacts with oxygen to form superoxide anion radical ($O_2^{\bullet-}$), which is converted into hydroxyl radical in the presence of iron through the Haber–Weiss reaction or forms peroxynitrite ($ONOO^-$) in the presence of nitric oxide ($^{\bullet}NO$) (Malisza

Abbreviations: ANP, atrial natriuretic peptide; AU, arbitrary unit; β-MHC, beta-myosin heavy chain; BNP, brain natriuretic peptide; cDNA, complementary deoxyribonucleic acid; DOX, doxorubicin; ECG, electrocardiogram; ESR, electron spin resonance; ET, ventricular ejection time; iNOS, inducible nitric oxide synthase; IO, iron overload; i.p., intra peritoneal; IR, ischemia/reperfusion; IVCT, isovolumic contraction time; IVRT, isovolumic relaxation time; IVS, inter ventricular septum; LVEDD, left ventricular end-diastolic diameter; LVEF, left ventricular ejection fraction; LVESD, left ventricular end-systolic diameter; LVMN, left ventricular mass normalized to body weight; LVPW, left ventricular posterior wall; mRNA, messenger ribonucleic acid; RONS, reactive oxygen and nitrogen species; RT-PCR, Reverse transcription polymerase chain reaction; SEM, standard error of the mean.

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and Hasinoff, 1995). In turn, these potent RONS induce lipid peroxidation and DNA damage (Muindi et al., 1984; Myers et al., 1982), thus promoting the death of cardiac cells by apoptosis or necrosis. Moreover, DOX-mediated RONS production may lead to the accumulation of iron in ferritin, and the DOX-iron complex impairs the DNA-binding activity of iron regulatory protein (IRP), leading to deregulation of iron homeostasis in cells (Gudjoncik et al., 2014). This process may lead to additional deleterious effects because iron is essential in several metabolic processes (Ammar el et al., 2011; Xu et al., 2005).

Although the involvement of iron in anthracycline-induced cardiotoxicity is suggested by studies in which iron chelators were shown to be cardioprotective (Dorr, 1996; Pouillart, 2004), some clinical trials led to disappointing results (van Dalen et al., 2008; Wouters et al., 2005). For now, the only iron chelator approved for the prevention of DOX-induced cardiotoxicity is dexrazoxane. However, this drug has been proved to prevent cardiotoxicity through several pathways (inhibition of topoisomerase IIβ, intracellular iron chelation, induction of hypoxia-inducible transcriptional factors). These multiple effects may explain why dexrazoxane is a more potent prophylactic agent than other iron chelators (Kalam and Marwick, 2013). In experimental models, iron loading was found to potentiate anthracycline cardiotoxicity in cardiomyocytes (Hershko et al., 1993; Link et al., 1996) and *in vivo* (Panjrath et al., 2007) as well as in hemochromatosis gene *Hfe*−/− mice (Miranda et al., 2003). However, the pharmacological interaction between iron and anthracyclines remains controversial and some authors found that DOX may paradoxically protect cardiomyocytes against iron-mediated cardiotoxicity (Corna et al., 2004), and that cardiomyocytes display a dose-dependent biphasic response to iron overload (Munoz et al., 2010).

The aim of our study was first to investigate *in vitro* the ability of dextran–iron to modify the DOX-induced deterioration of cell viability in cultured cancer cells (EMT-6) and in cardiomyocytes (H9c2). Secondly, we sought to investigate *in vivo* in mice, the impact of chronic tissue iron loading on DOX-induced nitro-oxidative stress and cardiotoxicity. We chose 20 days of daily 15 mg/kg dextran–iron loading since this iron formulation and dosing protocol was found to induce serum, hepatic and cardiac iron loading in C57Bl/6 mice (Moon et al., 2011; Wouters et al., 2005). We then deliberately chose a non-lethal dose of 6 mg/kg of DOX in order to reveal any potential deleterious effects of iron loading, and to be able to follow the evolution of myocardial function ten days after its injection. We evaluated iron status, nitro-oxidative stress levels and cardiac functional alterations. The major finding of our study was that, paradoxically, iron loading did not potentiate DOX cardiotoxicity in either cell cultures or mice, once again feeding the debate concerning the detrimental role of iron in DOX-induced cardiotoxicity.

Material and methods

Cell culture and treatments

The H9c2 embryonic rat heart-derived and the EMT-6 mouse mammary carcinoma cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA) (see Supplementary material online for details). All cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 4 mM L-glutamine, 100 U/mL penicillin, 100 U/mL streptomycin and 250 ng/mL amphotericin B in humidified air (5% CO_2) at 37 °C. Assays on cells were performed by COHIRO Biotechnology (Faculty of Medicine, Dijon, France).

Briefly, when the cultures reached 70–80% of confluence, cells were suspended in 0.05% (w/v) trypsin/0.53 mM ethylenediaminetetraacetic acid (EDTA) and plated at a density of 5000 cells/well in 96-well plates in medium containing 5% FBS and 2 mM L-glutamine. After 24 h, Iron–Dextran (Ferristat®) was added to achieve the final concentrations of 125, 250, 500 and 1000 μg/mL. Six hours after iron loading, cells were treated with or without 1 μM DOX (Adriblastin®) for 20 and 68 h of treatment for H9c2 cells, and with 2 or 10 μM of DOX for 20 h of treatment for EMT-6 cells. Cell viability was measured with the XTT assay, which is based on the ability of metabolically active cells to reduce tetrazolium salt XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxyanilide inner salt) to orange colored compounds of water-soluble formazan crystals *via* mitochondrial and extracellular dehydrogenases. The medium was removed and replaced with fresh medium without FBS and L-glutamine containing 0.15 mg/mL XTT solution, and the cells were further cultured in the CO_2 incubator at 37 °C for 4 h. The amount of formazan produced was detected by measuring absorbance at 499 nm, and a reference wavelength was used at 660 nm on an Infinite M200 Pro microplate reader (TECAN, Lyon, France). Cell damage was measured by the release of lactate dehydrogenase (LDH Kit, Promega, Charbonnières, France) into the extracellular medium. Cell viability and cell damage were expressed as a percentage of control, which was taken as 100%. In all experiments, four sets of wells were run and the experiment was repeated at least three times with different cell preparations.

Animals and treatments

Male C57Bl/6 mice (Charles River, L'Arbresle, France) aged 10 weeks were used. All animals received humane care and study protocols complied with the institution's guidelines. The investigation complied with Directive 2010/63/EU of the European Parliament and with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the local ethics committee (Comité d'Ethique de l'Expérimentation Animale, Université de Bourgogne, Dijon, France, protocol agreement number: 3211). Throughout the procedure, care was taken to prevent suffering and to ensure animal welfare, for instance, through improving the environment in cages. During the protocol, no animals became moribund and needed to be euthanized early or died from the treatment.

The experimental protocol is described in Fig. 1. To evaluate the effects of IO on DOX-induced cardiotoxicity, 32 male mice had echocardiography at D0; then, for 20 days (D1 to D20), they received a daily intraperitoneal (i.p.) injection of dextran/iron or saline solution. At D21 a second echocardiography was done and the mice were given or not given a single i.p. injection of doxorubicin (DOX, 6 mg/kg). Four groups were then constituted: a control group (CONT, n = 8), iron loading alone (IO, n = 8), DOX alone (DOX, n = 8) or the combination of iron and DOX (IO + DOX). At D30, all of the mice underwent

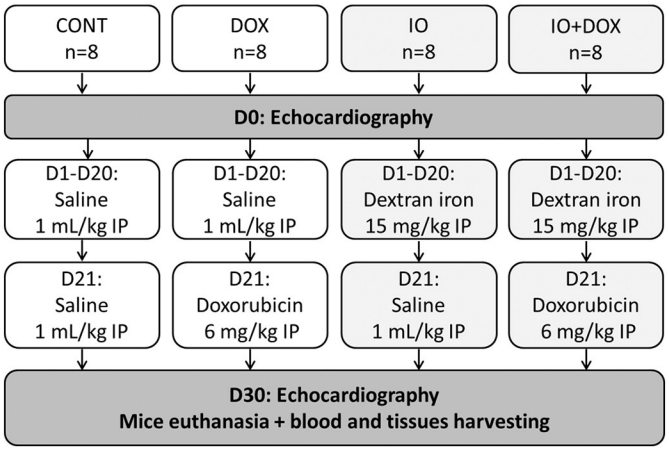

Fig. 1. Experimental design and study groups. At D0, 32 male mice underwent transthoracic echocardiography. From D1 to D20 mice received a daily i.p. injection of dextran/iron (15 g/kg) or saline solution, followed or not at D21, after a second echocardiography, by a single i.p. injection of doxorubicin (DOX, 6 mg/kg). Four groups were then constituted, control groups (CONT, n = 8), iron loading alone (IO, n = 8), DOX alone (DOX, n = 8) or the combination of iron and DOX (IO-DOX). At D30, all mice underwent echocardiography.

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