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Sex-related differential susceptibility to doxorubicin-induced cardiotoxicity in B6C3F$_1$ mice

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ABSTRACT

Sex is a risk factor for development of cardiotoxicity, induced by the anti-cancer drug, doxorubicin (DOX), in humans. To explore potential mechanisms underlying differential susceptibility to DOX between sexes, 8-week old male and female B6C3F$_1$ mice were dosed with 3 mg/kg body weight DOX or an equivalent volume of saline via tail vein once a week for 6, 7, 8, and 9 consecutive weeks, resulting in 18, 21, 24, and 27 mg/kg cumulative DOX doses, respectively. At necropsy, one week after each consecutive final dose, the extent of myocardial injury was greater in male mice compared to females as indicated by higher plasma concentrations of cardiac troponin T at all cumulative DOX doses with statistically significant differences between sexes at the 21 and 24 mg/kg cumulative doses. A greater susceptibility to DOX in male mice was further confirmed by the presence of cytoplasmic vacuolization in cardiomyocytes, with left atrium being more vulnerable to DOX cardiotoxicity. The number of TUNEL-positive cardiomyocytes was mostly higher in DOX-treated male mice compared to female counterparts, showing a statistically significant sex-related difference only in left atrium at 21 mg/kg cumulative dose. DOX-treated male mice also had an increased number of γ-H2A.X-positive (measure of DNA double-strand breaks) cardiomyocytes compared to female counterparts with a significant sex effect in the ventricle at 27 mg/kg cumulative dose and right atrium at 21 and 27 mg/kg cumulative doses. This newly established mouse model provides a means to identify biomarkers and access potential mechanisms underlying sex-related differences in DOX-induced cardiotoxicity.

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1. Introduction

Doxorubicin (DOX) is a commonly used chemotherapeutic drug for the treatment of both hematologic malignancies (e.g., leukemia and lymphoma) and solid tumors (e.g., breast, ovary, uterus, and prostate) (Carvalho et al., 2009; Octavis et al., 2012; Volkova and Russell, 2011). One of the most serious side effects from DOX treatment is dose-related, cumulative and irreversible cardiomyopathy, leading to congestive heart failure (Haq et al., 1985; Shan et al., 1996; Steinherz et al., 1991). A consensus amongst experts suggests that DOX-induced cardiotoxicity most likely involves multifaceted and complex biochemical pathways (Von Hoff et al., 1979). A plethora of reports identify roles for oxidative stress, down regulation of genes associated with contractile proteins, topoisomerase 2β mediated alterations in the transcriptomes that selectively affect oxidative phosphorylation, and p53-mediated apoptosis as a few of the many biological mechanisms for DOX-induced cardiomyopathy (Doroshow et al., 1980; Rajagopalan et al., 1988; Volkova and Russell, 2011; Zhang et al., 2012). Interestingly, studies identify gender as a clinically significant risk factor associated with DOX-induced cardiotoxicity (Hequet et al., 2004). Differences in cardiac oxidative stress, variations in multidrug-resistance enzymes, and body composition have all been suggested as underlying elements in sex-based differences and sensitivity to DOX treatment (Lipshultz et al., 1995). In prepubescent oncology patients, females are more vulnerable than males for developing DOX-induced cardiotoxicity (Krischer et al., 1997; Lipshultz et al., 1995; Trachtenberg et al., 2011). Conversely, in adult cancer patients, males are more susceptible than

Abbreviations: cTnT, cardiac troponin T; DAB, diaminobenzidine; DOX, doxorubicin; DOXol, doxorubicinol; DSB, DNA double strand breaks; HCT, hematocrit; Hgb, hemoglobin; IgG, immunoglobin G; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCs, mast cells; MCV, mean corpuscular volume; RBC, red blood cells; ROS, reactive oxidative species; RT, room temperature; TUNEL, terminal deoxynucleotidyl transferase (TdT) dUTP nick end labeling; WBC, white blood cells.

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age-matched females to DOX-induced cardiotoxicity as measured by fractional shortening (Hequet et al., 2004). Higher estrogen levels seem to provide a cardioprotective effect in women since a greater risk for developing DOX-induced cardiotoxicity exists in postmenopausal women compared with premenopausal women (Hershman et al., 2008).

Animal model systems are being used to advance knowledge of mechanisms underlying sex-related differential susceptibility to development of DOX-induced cardiomyopathy (Gonzalez et al., 2015; Moulin et al., 2015; Zhang et al., 2014). For example, adult spontaneously hypertensive male and ovariectomized female rats, receiving 1 mg/kg DOX for 9, 10, and 12 weeks show more severe cardiomyopathy and higher cardiac troponin T (cTnT) levels in serum compared with normal females, suggesting a likely role for estrogen in cardio-protection against DOX toxicity (Zhang et al., 2014). Another study, using a tumor-bearing spontaneous hypertensive rat (SHR/SST-2s) model system, demonstrates a dramatic influence of DOX treatment on development of cardiotoxicity in adult SHR/SST-2s male-, castrated male-, female-, and ovariectomized female- rats, showing the most severe cardiotoxic effects in male rats as measured by increased heart weight, higher cardiac lesion scores and increased serum levels of cTnT (Gonzalez et al., 2015). DOX treatment in this rat model system induced a dramatic decline in estrogen levels, to non-detectable levels in male animals when compared to saline (SAL) treated counterparts, further advancing the premise of a likely cardioprotective role for estrogen (Gonzalez et al., 2015). Furthermore, in adult Wistar rats, differential mitochondrial dysfunction and altered energy signaling pathways have been implicated in sex-based differences in DOX-induced cardiotoxicity (Moulin et al., 2015).

Another factor that may implicate differential susceptibilities between sexes is the therapeutic index for administering anticancer drugs, like DOX. For example, higher doses of some therapeutic drugs (e.g., cisplatin, with a relatively high therapeutic index) show better efficacy in women without increasing toxicity, while others given at a higher dose in women (e.g., 5-flurouracil, with a relatively low therapeutic index) show increased toxicity, and no improvement in efficacy (Schmetzer and Florcken, 2012). Data describing sex-related variations in the therapeutic index for DOX is limited. Following administration to cancer patients, DOX is rapidly taken up by most tissues (Speth et al., 1988). Pharmacokinetic information indicates that at least 50% of DOX (or one of its metabolites) is excreted via bile and urine from the body, normally within a span of 5 days (Speth et al., 1988). Doxorubicinol (DOXol), a major metabolite of DOX, is reported to be more cardiotoxic than DOX in animal models (Cusack et al., 1993; Olson et al., 1988; Sacco et al., 2003). Despite having a mean elimination half-life of 13–40 h in cancer patients, detectable levels of DOX and/or DOXol are found in heart tissue for prolonged periods after cessation of treatment, while negligible levels are found in skeletal and smooth muscle organs (Cummings and Smyth, 1988; Del Tacca et al., 1985; Olson et al., 1988; Rodvold et al., 1988; Stewart et al., 1993). Differential accumulation of these compounds in myocardial tissue could be a contributing factor that modulates gender-related sensitivity to DOX cardiotoxicity. Definitive information regarding DOX pharmacokinetics in both male- and female- hearts could help to resolve this question.

Building on an existing mouse model of DOX-induced chronic cardiotoxicity developed in adult male B6C3F$_1$ mice; the present study describes differential susceptibility to DOX between male- and female-mice. This newly established mouse model demonstrated discernable differences and degrees to which cardiac injury and pathologies occurred in adult male B6C3F$_1$ mice compared with females following DOX treatments. Furthermore, apoptotic changes and DNA damage in cardiomyocytes from various regions of the heart were greater in DOX-treated male mice compared with DOX-treated females. This mouse model provides an important means for examination of the molecular basis of differential susceptibility to cardiotoxicity, such as differences in cardiac oxidative stress and DOX pharmacokinetics between the sexes. Such information will be useful for developing sex-specific susceptibility or cardiac injury biomarkers for early identification, assessment, and monitoring of DOX-induced cardiotoxicity, as well as in promoting new treatment regimens and/or interventions to minimize cardiotoxicity and maximize the efficacy of DOX in the clinic.

2. Material and methods

2.1. Animal husbandry

Male and female B6C3F$_1$ mice were purchased as weanlings from Jackson Laboratories (Bar Harbor, ME) and were allowed 3–7 days to acclimatize in the animal facility at the National Center for Toxicological Research (NCTR). Animals were raised in a pathogen-free environment at the NCTR and treated according to the Institutional Animal Care and Use Committee guidelines. Mice were housed individually in standard polycarbonate cages with hardwood chip bedding and animal rooms were maintained at 23 °C with a relative humidity of 50%. The animals were conditioned to a 12/12-h light/dark cycle and had *ad libitum* access to NIH-41 IR diet (LabDiet, Richmond, IN) and water.

2.2. Animal treatments

Beginning at 8 weeks of age, male and female mice each received a dose of 3 mg/kg body weight DOX intravenously via the tail vein once weekly for 6, 7, 8, or 9 consecutive weeks, resulting in cumulative DOX doses of: 18 mg/kg (53.3 mg/m^2), 21 mg/kg (61.2 mg/m^2), 24 mg/kg (71.0 mg/m^2), or 27 mg/kg (79.9 mg/m^2), body weight respectively. Animal doses expressed in units of mg/kg were converted to human equivalent doses in mg/m^2 using the formula described in the Guidance for Industry document (CDER, 2002). Doxorubicin (LKT Laboratories, St. Paul, MN) was dissolved in sterile 0.9% saline (Sigma-Aldrich, St. Louis, MO) to a final concentration of 1.5 mg/ml. Treated animals received 2 μl/g body weight of the dose solution. Control mice received an equivalent volume of sterile 0.9% SAL via the tail vein once weekly for 6, 7, 8, or 9 weeks. Each DOX treatment group consisted of 5 mice per group per sex, whereas each SAL treatment group consisted of 4 mice of each sex. All experimental procedures were performed in compliance with the Guide for the Care and Use of Laboratory Animals. The animals were observed daily for abnormal signs, weighed weekly and food consumption was measured twice weekly during the course of the study.

2.3. Collection and processing of tissues

One week before initial dosing, blood was collected from the submandibular vein of each mouse into Microtainer® tubes coated with EDTA (BD Biosciences, Franklin Lakes, NJ) for the measurements of basal levels of hematological parameters (red blood cell (RBC) count, hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), white blood cell (WBC) count, and platelets). Every week during the course of the treatments, blood was collected from 2 to 3 mice from each DOX- or SAL-treatment groups to monitor changes in hematological parameters. One week following the last dose, mice were anesthetized by inhalation of 2.0% isoflurane mixed with 100% oxygen using an Euthanex E-Z Anesthesia system (Euthanex, Palmer, PA) and blood was collected by retro-orbital puncture into Microtainer® tubes coated with EDTA. A small portion of whole blood was used for measurement of hematology parameters. The remaining blood was immediately centrifuged at 1000 $\times g$ for 10 min at 4 °C to separate plasma for measurement of cardiac troponin T (cTnT) concentrations. Following retro-orbital bleeding, mice were euthanized by exsanguination and the heart was immediately excised and separated from the pericardium. The left atrium, right atrium, and