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Altered mitochondrial epigenetics associated with subchronic doxorubicin cardiotoxicity

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ABSTRACT

Doxorubicin (DOX), a potent and broad-spectrum antineoplastic agent, causes an irreversible, cumulative and dose-dependent cardiomyopathy that ultimately leads to congestive heart failure. The mechanisms responsible for DOX cardiotoxicity remain poorly understood, but seem to involve mitochondrial dysfunction on several levels. Epigenetics may explain a portion of this effect. Since mitochondrial dysfunction may affect the epigenetic landscape, we hypothesize that this cardiac toxicity may result from epigenetic changes related to disruption of mitochondrial function. To test this hypothesis, eight-week-old male Wistar rats (n = 6/group) were administered 7 weekly injections with DOX (2 mg kg^{-1}) or saline, and sacrificed two weeks after the last injection. We assessed gene expression patterns by qPCR, global DNA methylation by ELISA, and proteome lysine acetylation status by Western blot in cardiac tissue from saline and DOX-treated rats. We show for the first time that DOX treatment decreases global DNA methylation in heart but not in liver. These differences were accompanied by alterations in mRNA expression of multiple functional gene groups. DOX disrupted cardiac mitochondrial biogenesis, as demonstrated by decreased mtDNA levels and altered transcript levels for multiple mitochondrial genes encoded by both nuclear and mitochondrial genomes. Transcription of genes involved in lipid metabolism and epigenetic modulation were also affected. Western blotting analyses indicated a differential protein acetylation pattern in cardiac mitochondrial fractions of DOX-treated rats compared to controls. Additionally, DOX treatment increased the activity of histone deacetylases. These results suggest an interplay between mitochondrial dysfunction and epigenetic alterations, which may be a primary determinant of DOX-induced cardiotoxicity.

1. Introduction

Doxorubicin (Adriamycin®, DOX) was one of the first anthracyclines to be isolated from strains of Streptomyces actinobacteria in the 1960s (Arcamone et al., 2000). Since its clinical introduction in the 1970s, DOX has remained one of the most frequently prescribed components in several currently used chemotherapy drug regimens for treating breast, ovarian and gastric carcinomas, sarcomas, leukemias, non-Hodgkin's and Hodgkin's lymphoma, multiple myeloma and many other cancers (Simunek et al., 2009; Sterba et al., 2013). The impact of anthracycline-based therapies is particularly noteworthy in pediatric oncology, where the 5-year survival rate for childhood cancer has increased from around 30% in the 1960s to over 70% in the modern era. Estimates are that over 50% of childhood cancer survivors have received some form of anthracycline treatment (Simunek et al., 2009; Sterba et al., 2013).

Despite having over 40 years of extensive clinical utilization, DOX mechanism of action remains a matter of controversy, as novel mechanisms are continuously being proposed. The cytostatic and cytotoxic actions of DOX in cancer cells have been attributed to various mechanisms, most often: 1) DNA intercalation, 2) topoisomerase II inhibition, 3) generation of free radicals with consequent induction of oxidative stress and 4) apoptosis induction – although the latter probably is an outcome, and not a cause of the aforementioned events (Gewirtz, 1999). Novel alternative mechanisms proposed include inhibition of DNA methylation enzymes (Yokochi and Robertson, 2004) and nucleosome destabilization induced by chromatin torsional stress (Yang et al., 2014). This array of antitumor mechanisms is likely to underlie the broad spectrum of therapeutic activity displayed by DOX.

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As is the case for every anticancer agent, DOX is a double-edged sword due to its toxic side effects in healthy tissue – cardiac muscle, in particular. Administration of DOX commonly results in readily reversible short-term acute effects such as nausea, diarrhea, alopecia and electrocardiography alterations (Carvalho et al., 2009; Sterba et al., 2013). Chronic cardiotoxic effects, on the other hand, are of much greater concern. Cumulative doses exceeding 500–550 mg/m^2 result in a clinically unacceptable risk of developing congestive heart failure (CHF) (Lefrak et al., 1973), thus severely limiting the clinical utility of this compound, which may persist until as many as 20 years after cessation of treatment (Steinherz et al., 1991).

The proposed mechanisms to account for this cardiomyopathy are as diverse as the aforementioned antineoplastic mechanisms (Carvalho et al., 2014), albeit entirely independent. Mitochondrial interactions are among the most commonly implicated and, consequently, best described mechanisms. DOX has been known to accumulate in cardiac mitochondrial membranes since the early 80s (Goormaghtigh et al., 1980; Nicolay et al., 1986; Peters et al., 1981). Since then, numerous studies have identified several forms of mitochondrial dysfunction and concomitant apoptotic signaling across various *in vitro* and *in vivo* experimental models (Carvalho et al., 2014). None of these studies, however, addressed the time course of events in the development of persistent cardiotoxicity.

As described above, DOX toxicity persists during an extended period of time (Steinherz et al., 1991). This phenomenon is characteristic of DOX cardiotoxicity, although it is mostly unexplored in the literature. Deleterious alterations in cardiac mitochondrial function, including oxygen consumption, free radical generation, decreased calcium loading capacity and altered mtDNA copy number and gene expression profile (within the studied time frame) (Berthiaume and Wallace, 2007; Richard et al., 2011; Serrano et al., 1999; Zhou et al., 2001), which again supports the notion that DOX toxicity in the myocardium is persistent and irreversible in nature. Clearly, long-term persistence of DOX cardiotoxicity has a large impact in survivors of childhood cancer (Bar et al., 2003), since it may lead to the appearance of later cardiac alterations during stressful events (including pregnancy) (Johnson et al., 1997).

Among the several hypotheses for this long-term DOX toxicity, the persistent alterations of gene expression in DOX-treated animals theory suggests that an epigenetic mechanism may be operating in promoting long-term DOX toxicity. Published data demonstrate that DOX causes a persistent and irreversible alteration of mitochondrial metabolism and gene expression (Berthiaume and Wallace, 2007; Richard et al., 2011). Interestingly, mitochondrial metabolism, as controlled by environment (*i.e.* nutrient availability) appears to have a critical role in the epigenomic landscape of nuclear DNA. When the energy supply is abundant, enzymes use mitochondrial-derived ATP and acetyl coenzyme A (Ac-CoA) to phosphorylate and acetylate chromatin, increasing gene expression. The opposite occurs when metabolism is decreased (Wallace and Fan, 2010). In other words, the notion that mitochondrial production of ATP, acetylcarnitine, and Ac-CoA directly impacts epigenetic regulation is a major framework for toxicological studies based on interference of mitochondrial bioenergetics. Toxicants that directly disturb mitochondrial function may deprive the accessibility of the nuclear DNA/histones to a source of acetyl groups and phosphate. In fact, DOX has been described to disturb creatine-phosphate shuttles (Tokarska-Schlattner et al., 2006), as well as inhibit carnitine palmitoyl transferase I (CPT) and/or deplete its substrate L-carnitine (Tokarska-Schlattner et al., 2006). Furthermore, DOX may also disturb the patterns of DNA/histone methylation. Depressed expression appears to be particularly focused on genes coding for enzymes participating in fatty acid beta-oxidation and mitochondrial ATP production (Berthiaume and Wallace, 2007), which suggests impaired mitochondrial Ac-CoA, acetylcarnitine, and ATP production. Furthermore, experiments using ρ0 cells support the notion that mitochondrial metabolic integrity is a requirement for proper nuclear DNA methylation patterns (Smiraglia et al., 2008). Moreover, DOX cardiotoxicity in Wistar rats was decreased by co-administrating S-adenosylmethionine (SAM), the major biological methyl donor (Russo et al., 1994). All together, these studies strengthen the hypothesis that alterations of epigenetic landscape may occur during DOX toxicity through interference with cell and mitochondrial metabolism. The present paper aims to explore the involvement of crosstalk between mitochondrial dysfunction and epigenetics in long-term DOX cardiotoxicity. We hypothesize that alterations to the nuclear epigenetic landscape lead to long-term changes in gene expression and contribute to the long lasting mitochondrial toxicity manifested as decreased mitochondrial capacity and gene expression.

2. Materials and methods

2.1. Animals and treatment protocol

Animal handling was performed in accordance with the European Directive on the protection of animals used for scientific purposes (2010/63/EU). The procedures were approved by the CNC Committee for Animal Welfare and Protection. Male Wistar-Han rats were purchased form Charles River Laboratories (Barcelona, Spain) at 7 weeks of age and acclimated for one week in local animal house facilities (CNC – Faculty of Medicine, University of Coimbra, Coimbra, Portugal). Two animals were housed per type III-H cage (Tecniplast, Italy) with irradiated corn cob grit bedding (Scobis Due, Mucedola, Italy) and environmental enrichment, maintained in controlled environmental requirements (22 °C, 45–60% humidity, 15–20 air changes/hour, 12 h artificial light/dark cycle, noise level < 55 dB) and free access to standard rodent food (4RF21 GLP certificate, Mucedola, Italy) and HCl-acidified water (pH 2.6).

Experimental manipulation was initiated with 8 weeks old rats weighing 220–260 g. Animals were randomly assigned to one of two experimental groups: saline (SAL)-treated (n = 6) and DOX-treated (n = 6). Rats received seven weekly subcutaneous injections of DOX (2 mg kg^{-1}), dissolved in normal saline (0.9% [w/v] NaCl), or an equivalent volume of SAL solution. All animals were injected and weighed during the light phase of the cycle. Animals were euthanized by cervical dislocation followed by decapitation two weeks after the last injection (Supplementary Fig. S1). Unlike most previous subchronic DOX treatment works (Machado et al., 2010; Pereira et al., 2012, 2016; Serrano et al., 1999), we opted to provide one extra week after DOX treatment to allow for a better assessment of the long-term effects in the absence of the drug.

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from heart and liver tissue using the Aurum Total RNA Mini Kit (Bio-Rad, Hercules, CA, USA), according to the manufacturer's specifications. Thereafter, RNA quality and integrity was ascertained by visualization of the 28S/18S rRNA band pattern using an Experion Automated Electrophoresis System (Bio-Rad). Total RNA was quantified spectrophotometrically at A260 nm using a NanoDrop 2000 spectrophotometer (Thermo Scientific) and stored at −80 °C until use. First-strand complementary DNA (cDNA) synthesis was performed using the iScript cDNA synthesis kit (Bio-Rad), according to the manufacturer's specifications.

2.3. DNA extraction

Total genomic DNA for mtDNA and m5C quantification was extracted from heart and liver tissue using the PureLink™ Genomic DNA Mini Kit (Invitrogen), according to the manufacturer's instructions. The extracted DNA samples were quantified spectrophotometrically at A260 nm using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) and stored at −80 °C until use.