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Doxorubicin-Induced Cardiotoxicity: From Mechanisms to Development of Efficient Therapy

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Abstract

First isolated in the early 1960s, doxorubicin (DOX) is among the most effective anticancer agents ever developed. DOX has been used mainly for the treatment of breast cancer, solid tumors in children, soft tissue sarcomas, and aggressive lymphomas. However, the use of DOX may have dose-dependent cardiotoxic effects that generate changes in myocardial structure, which can develop into severe and irreversible cardiomyopathy. Here, we describe the incidence of DOX-induced cardiotoxicity (DIC); the progress made over the past four decades in understanding the molecular mechanisms of the pathogenesis of acute and chronic DIC; the current strategies for heart protection; and the major breakthroughs and challenges in basic and clinical research to the development of efficient targeted therapy for DIC.

Keywords: doxorubicin, chemotherapy, cardiotoxicity, mechanisms, pathogenesis, heart protection, targeted therapy

1. Introduction

Cardiomyopathy induced by doxorubicin (DOX) is considered an extremely serious adverse effect of oncologic treatment. It is known that this disease significantly affects the quality of patients’ life who survived cancer, especially children. Since its discovery, several molecular mechanisms have been proposed to understand the pathogenesis of acute and chronic DOX-induced cardiotoxicity (DIC), including oxidative stress, iron metabolism, Ca²⁺ homeostasis dysregulation, sarcomeric structure alterations, gene expression modulation, and apoptosis. Based on these mechanisms, different strategies have been developed in order to protect the
heart during cancer treatment, including the administration of iron-chelating antioxidants and adrenergic receptor agonists. However, the use of these drugs is limited due to their adverse side effects as well as the loss of beneficial cardiac effects years after the end of the treatment. Therefore, the development of new therapies has been a great challenge for the scientific community. In this context, a new emergent strategy is cell therapy. Considering that DOX causes cardiomyocyte death, the transplant of autologous cardiomyocytes obtained through the differentiation of human induced pluripotent stem cells (iPSC) is a viable option for cardiac repair and a promising therapeutic strategy for the treatment of cardiovascular diseases, including DIC.

2. Doxorubicin

DOX (also known as adriamycin) was isolated in the early 1960s from the pigment-producing bacterium *Streptomyces peucetius var. caesius*, along with daunorubicin (DAU, also known as daunomycin and rubidomycin), and belongs to the family of anthracyclines [1–3]. Until now, DOX remains among the most largely prescribed and effective antineoplastic agents ever developed for the treatment of a variety of adult and pediatric cancers [3–5]. Whereas DAU has been used against acute lymphoblastic and myeloblastic leukemias, DOX has been used against breast cancer, soft tissue sarcomas, childhood tumors (e.g., Wilms’ tumor), leukemias, Hodgkin’s and non-Hodgkin’s lymphoma, and many other cancers [4, 5].

The minor differences in the chemical structure between DOX and DAU are responsible for the different spectrums of activity of these drugs. The side chain of DOX terminates with primary alcohol, while that of DAU terminates with a methyl group [3, 6]. Unfortunately, in addition to its potent antitumor effect, the use of DOX has been hampered by conventional toxicities (hematopoietic suppression, nausea, vomiting, extravasation, and alopecia), development of resistant tumor cells or toxicity in healthy tissues, especially with serious cardiac toxicity manifested by congestive cardiomyopathy [4, 7]. Over time, more than 2000 analogs were developed in an attempt to reduce the adverse effects of DOX and DAU. However, few analogs have reached the stage of clinical development and approval, such as epirubicin (EPI) and idarubicin (IDA), with DOX- and DAU-like spectrums, respectively [6]. Despite the development of new components, replacing DOX does not eliminate the risk of developing cardiotoxicity [6, 7]. Thus, DOX continues to be considered as a first-line antineoplastic drug [7].

2.1. DOX-induced cardiotoxicity: clinical aspects

Since the late 1970s, DOX-induced cardiotoxicity (DIC) has been recognized as a complication of chemotherapy [5]. The first case report in the literature was that of a 23-year-old patient with osteosarcoma, who was treated for 9 months with DOX. One month after the end of treatment, the patient died due to development of congestive heart failure [8]. A second report describes the case of an 11-year-old patient, also with osteosarcoma, who died 9.5 years after the end of chemotherapy with DOX as a result of progressive heart failure with late severity
In 1991, long-term cardiotoxic effects were identified in patients with acute lymphoid leukemia in childhood [10]. Patients with childhood cancer and those treated with DOX have a high risk of developing symptomatic cardiac events at an early stage, and this risk remains high within 30 years after treatment. In addition, it is estimated that one in eight DOX-treated patients will be afflicted with severe cardiac disease [11].

DIC manifests in several forms, ranging from asymptomatic electrocardiography (ECG)-changes to decompensated cardiomyopathy characterized by decreased left ventricular ejection fraction [4, 7]. According to their clinical manifestation, these cardiotoxic events can be classified into three types: (1) acute, occurring during or immediately after treatment; (2) early-onset chronic progressive cardiotoxicity, occurring within 1 year after exposure to chemotherapeutic treatment; and (3) late-onset chronic progressive cardiotoxicity, occurring 1 or more years after the end of treatment [11].

Acute cardiotoxicity is characterized by depression of myocardial contractility that may be reversible within 1 week when discontinuing the DOX treatment [12, 13]. In some patients, complications have already been described, such as hypotension; pericarditis; myocarditis; supraventricular, ventricular, or sinus (more common) tachycardia; ST-T wave changes; decrease in QRS complex; prolongation of QT interval; and increase in serum levels of brain natriuretic peptide and cardiac troponin [3, 12–14]. However, this type of cardiotoxicity is very rare and affects less than 1% of patients [12].

Early-onset chronic progressive cardiotoxicity is characterized by systolic or diastolic ventricular dysfunction within 1 year after the completion of DOX treatment. It can be progressive and occurs in 5–35% of the cases [11, 14, 15]. In the majority of adult patients, early cardiotoxicity is related to the development of a chronic dilated cardiomyopathy, with a decrease in the mass and wall of left ventricle. In the pediatric patient, in addition to chronic dilated cardiomyopathy, restrictive cardiomyopathy characterized by increase in the wall stiffness of the left ventricle cavity may also occur in isolated moments [11–13]. The typical manifestation of these cardiomyopathies is the progressive reduction of the ejection fraction [13]. Other events, including severe electrical conduction changes, damage to cardiac valves, and/or depression of contractility may also be observed [15].

Finally, late-onset chronic progressive cardiotoxicity is characterized by cardiac dysfunction after a latency period of 1 or more years following the completion of DOX treatment [12, 13]. In this type of cardiotoxicity, there is a period during which the patient is asymptomatic (normal cardiac function). After that, chronic dilated and/or restrictive cardiomyopathy can be manifested with subsequent development of congestive heart failure. In this case, mortality rate is more than 50% [3, 12, 13, 15].

### 2.2. Mechanisms of cardiotoxicity

Despite almost 60 years of research, the mechanisms to explain DIC are not completely understood. It seems to be a multistep process, with different potential pathways involved that leads to cardiomyocyte death [11, 16, 17]. Until now, the main mechanisms that have been proposed by various research groups include oxidative stress, iron metabolism, Ca²⁺...
homeostasis dysregulation, sarcomeric structure alterations, gene expression modulation, and apoptosis [4, 13, 16, 17].

2.2.1. Oxidative stress

Since the discovery of DOX, oxidative stress is the most frequently proposed mechanism to explain the complex pathophysiology of DIC [3, 5, 16]. The myocardium injury evidenced by lipid peroxidation occurs as a result of the increase of the reactive oxygen species (ROS) production, including superoxide (O$_{2}^{-}$) and hydroxyl radicals (OH) as well as other non-radicals such as hydrogen peroxide (H$_2$O$_2$), singlet oxygen (O$_2$), etc. [3, 4, 11, 17, 18]. Unlike other tissues, the heart is extremely prone to oxidative damage, at least in part, due to lower levels of antioxidant enzymes such as peroxidase, catalase, and superoxide dismutase. In addition, the chemical structure of DOX contains quinone groups that can be reduced to a semiquinone, an unstable metabolite which can react with molecular oxygen (an electron acceptor) and rapidly revert to the parent compound. This redox cycle leads to the formation of superoxide anion radicals within mitochondria, causing cardiotoxicity [11, 16–20].

The mitochondria have been identified as the main subcellular organelles injured in the heart by DIC [4, 17]. DOX is a cationic drug that binds with high affinity to cardiolipin (a phospholipid) forming nearly irreversible complex in the mitochondrial inner membrane [17, 21]. It is important to know that cardiolipin is required for the proper functioning of the electron-transport chain proteins. In this context, evidence suggests that DOX disrupts the cardiolipin-protein interface, causing more superoxide anion radicals formation [4, 22]. As a result, ROS can induce different forms of cardiomyocyte death (apoptosis or necrosis) [17]. Furthermore, the reduction of mitochondrial function causes energetic metabolism change evidenced by a decrease of the adenosine triphosphate (ATP) production, which may contribute to abnormal contraction and relaxation in the failing heart [4, 11, 23].

Other forms of DOX-induced ROS generation in the myocardium include nitric oxide synthases (NOS) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidases pathways. These enzymes interact with DOX and induce oxidative stress [4, 16, 17].

NOS are a group of enzymes responsible for the nitric oxide (NO) production from L-arginine and oxygen [24]. The NO generation is altered by the direct binding of DOX to endothelial NOS (eNOS) reductase domain, leading to the reduction of the DOX semiquinone radical, which reacts with oxygen and produces superoxide. There is evidence to suggest that, in low DOX concentrations, eNOS signaling is the main pathway for DOX reduction. In addition, the increase of DOX-eNOS interaction completely modifies normal functioning of the enzyme (NO production) and transforms it into a potent superoxide generator [25]. DOX also affects NOS signaling by increasing eNOS transcription and protein activity in bovine aortic endothelial cells (BAEC). In this study, BAEC were pretreated with eNOS antisense oligonucleotides or antioxidants and the results showed apoptosis decrease [26]. Recently, an in vivo study has shown that the pretreatment with folic acid (FA, a modulator of eNOS) prevented DOX-induced increases in superoxide anion and attenuated DOX-induced decreases in superoxide dismutase, eNOS phosphorylation, and NO production [27]. Another study
showed a decrease in ROS generation, preservation of cardiac function, and reduction of mort-
tality rate after acute and chronic DOX administration in the eNOS knock-out (eNOS−/−) mice model, whereas cardiomyocyte-specific eNOS overexpression intensified the pathological response to DOX in the heart [28]. In all, these studies demonstrate the importance of eNOS signaling in DIC.

Recent evidence suggests that the other isoform called inducible NOS (iNOS) is also involved with DOX-induced oxidative stress. In some studies, iNOS transcription and expression are increased in mouse and rat hearts and isolated cardiomyocyte after DOX treatment [29–31]. In iNOS knock-out (iNOS−/−) mice model, cell death and nitrotyrosine (NT) formation induced by DOX were mitigated. The same results were observed when selective iNOS inhibitors such as 5,5′-[(1,3-phenylene-bis(1,2-ethanediyl))bis-isothiourea (1,3-PB-ITU) and L-N6-(1-iminorhyl)-lysine (L-NIL) were administered. In this study, DIC occurs due to the generation of peroxynitrite, a potent oxidant which generates secondary free radicals, including nitrogen dioxide and carbonate radical [30]. It is possible that the reduction of the peroxynitrite production using specific antioxidant(s) is a viable strategy for the decrease of DIC. In support of this view, the significant increase of superoxide radical and peroxynitrite induced by DOX observed in isolated cardiomyocytes was blunted after treatment with vitamin C (Vit C). These results suggest that Vit C provides cardioprotection by reduction of oxidative/nitrosative stress [31]. Altogether, these works thus also highlight the importance of iNOS signaling in DIC.

The activity of the third isoform, neuronal NOS (nNOS), in DOX-induced oxidative stress is poorly understood. It appears that the flavin domain is involved with DOX reduction [32]. Nevertheless, no changes were observed in nNOS transcription and protein activity after the treatment of DOX [30]. Therefore, further studies will be needed to elucidate the role of NOS isoforms as well as the therapeutic potential of their pharmacological targeting in DOX-dependent heart disease.

In relation to NADPH oxidases, also known as NOXs, recent work has identified these enzymes as important sources of myocardial ROS [33]. NADPH oxidase is a multicompo-
ent complex that consists of membrane-bound cytochrome b-558, which is a heterodimer of gp91phox and p22phox, cytosolic regulatory subunits p47phox and p67phox, and the small GTP-binding protein Rac1 [34]. These enzymes mediate the transfer of one electron from NADPH to quinone DOX, leading to DOX semiquinone radical. As result, they can produce superoxide similar to NOSs. The semiquinone radical also reacts with hydrogen peroxide generating hydroxyl radicals [35]. An in vitro study using NADPH oxidase inhibitors (diphe-
nylodionium and apocynin) on H9c2 cells showed that DOX-induced apoptosis was miti-
gated, demonstrating NADPH oxidase is also involved in the development of cardiac toxicity induced by DOX [36]. Furthermore, there is accumulating evidence to support an important role for Nox2 NADPH oxidase (one of the seven different NADPH oxidase isoforms) in DIC, identified using Nox2-deficient (Nox2−/−) or gp91phox knock-out (gp91−/−) mice [33, 37–39]. DOX-induced cardiomyocyte apoptosis and atrophy, interstitial fibrosis, leukocyte infiltration, and cardiac dysfunction in wild-type (WT) mice were attenuated in Nox2−/− mice [33, 39]. DOX-induced superoxide production was also mitigated in this animal model [39].
Recently, Rac1 has been reported to be a key regulator of oxidative stress due to its ability to bind and activate the NADPH oxidases [34]. In this context, a study showed that the deletion of Rac1 (a subunit of the NADPH oxidases complex) in cardiomyocytes impairs DOX-induced NADPH oxidases activation, ROS generation, DNA fragmentation and apoptosis, and improves cardiac function [40]. The same results were observed when NSC23766, a RAC inhibitor, was administered. In contrast, the overexpression of Rac1 exacerbated DIC [41]. Therefore, Rac is extremely important for the regulation of DIC by NADPH oxidase/ROS-dependent pathway.

Patient’s genetic susceptibility is another factor that has been considered extremely important for the understanding of NADPH oxidases-dependent cardiotoxicity. Single-nucleotide polymorphisms (SNPs) in one of the subunits of the NADPH oxidases complex have been identified in non-Hodgkin lymphoma patients. After the treatment with DOX, these patients developed acute arrhythmias and congestive heart failure. For example, the presence of SNP variants in NADPH oxidase subunit NCF4 and in the p22phox and Rac2 subunits were linked with the development of chronic and acute DIC, respectively [36]. Thus, detection of the genetic polymorphisms in NADPH oxidases complex may help to identify patients who have higher risk to develop DIC.

2.2.2. Iron metabolism

It is reported that DOX is able to alter iron metabolism due to its strong affinity for this metal, thereby forming iron-DOX complexes which, in turn, react with oxygen and trigger ROS production [42]. Thus, the researchers believed that only oxidative stress was responsible for the cardiotoxicity induced by iron-DOX complexes. However, in physiological conditions, there would not be enough free iron to interact with DOX to the extent necessary to cause cardiomyopathy [6]. On the other hand, another theory suggests that the effect of DOX on iron metabolism occurs due to the interference of this drug in the activity of proteins that transport and bind intracellular iron. For example, one of the mechanisms involves the doxorubicinol (DOXol), a metabolite of DOX, which removes iron from the catalytic Fe-S cluster of the cytoplasmic aconitase (also called iron regulatory protein 1; IRP-1), converting this enzyme to a null protein. Consequently, there is an increase in the stability of transferrin mRNA and preventing translation of iron sequestration proteins. As a result, reduction of IRP-1 causes an increase in free iron, which can lead to free radical production [43, 44]. Furthermore, a recent work reports that DOX can also interact with iron-responsive elements (IREs) of the ferritin heavy and light chains. It is known that ferritin operates as an iron transporter, reducing free iron within the cell. Accordingly, disruption of this protein eventually results in increased free iron, which in turn causes myocardium injury [45]. Another work showed iron-overload, mitochondrial damage, and mortality after DOX treatment in mice depleted of the iron regulatory gene HFE (also known as human hemochromatosis protein). The HFE protein is responsible for the regulation of circulating iron uptake [46]. Therefore, free iron accumulation within the myocardium after DOX treatment seems to be the major determinant of DIC [20].

It is important to recognize that patients undergoing chemotherapy are submitted blood transfusions and iron supplementation due to abnormal losses and nutritional status deficient,
respectively. The fact is that these procedures modify body iron stores. In addition, adult and pediatric patients with leukemia can develop a significant level of iron-overload during, and as result of, chemotherapy [46]. Thus, it is possible that the reduction of iron levels is an effective strategy to prevent DOX-induced cardiomyopathy.

2.2.3. Calcium homeostasis dysregulation

The precise control of calcium levels during the contraction-relation cycle in cardiomyocytes is extremely important for normal beat-to-beat contractile activity [47]. Unfortunately, many studies suggest that calcium homeostasis dysregulation has a major role in the pathogenesis of DIC. To date, severe mechanisms have been proposed that are responsible for an increase in calcium intracellular concentrations [4, 16]. One of the mechanisms is related to DOX metabolism, which generates a toxic metabolite, DOXol, through a reduction of its carbonyl group, capable of inhibiting the sodium-calcium exchanger channel [48]. The sodium/potassium pump of the sarcolemma is also affected by DOXol, which disrupts the sodium gradient needed for calcium to flow into the sarcolemma of a cardiomyocyte [49]. Consequently, there is an imbalance in the energetics of the myocardium and diminished systolic function [48]. Furthermore, it is reported that this secondary metabolite is more difficult to eliminate from the cardiomyocyte than the parent drug [50]. Thus, DOXol accumulation contributes significantly to the dysregulation of calcium homeostasis, leading to myocardial damage.

Moreover, normal calcium homeostasis is altered by ROS and hydrogen peroxide via disruption of normal sarcoplasmic reticulum function. This is accomplished by inhibiting the Ca\(^{2+}\)-ATPase pumps, caused by reducing the expression of SERCA2a mRNA levels and/or the direct activation of the ryanodine calcium-release channels themselves [51, 52]. In addition, a study suggests that DOX induces calcium release from the sarcoplasmic reticulum due to increasing the frequency of opening of these channels [52]. At the same time, DOX induced the inhibition of sodium-calcium channels in the plasma membrane as well as increased L-type calcium channel activation [53, 54]. DOX has also been shown to decrease the calcium storage capacity of mitochondria by specifically activating the selective CsA-sensitive calcium channel, exacerbating the calcium-overload [49]. As result, an increase of calcium cytoplasmic concentrations occurs, leading to mitochondrial dysfunction and apoptosis [55]. Therefore, the preservation of calcium homeostasis is essential to prevent DOX-induced cardiomyopathy.

2.2.4. Sarcomeric structure alterations

DIC is also accompanied by disarray and loss of myofilaments of the sarcomere. Titin is a giant protein and a key component of the cardiac sarcomeres, extending from the M-line to the Z-disk. This protein has multiple functions, from structural to regulatory [56]. Recent studies have shown that the loss of integrity or function of titin is directly related to the development of dilated cardiomyopathy [57, 58]. It is known that DOX induces rapid degradation of titin through the activation of proteolytic pathways, leading to an imbalance in the energetics of the myocardium. Furthermore, studies have shown that the degradation of titin also occurs by the activation of calpains (calcium-dependent proteases) and reported that the inhibition of this protein is responsible for preserving cardiac function after DOX treatment [59].
Another study showed that the depletion of the cardiac ankyrin repeat protein (CARP), which are important in negative regulation of cardiac genes expression, leads to marked sarcomeric disarray [60]. Taken together, these studies thus also highlight the importance of sarcomeric structure stability to prevent DIC. It is necessary to recognize that other proteins are essential for sarcomeric cytoskeleton such as α-actinin, myomesin, and nebulin, and further studies should be performed to verify the DOX effect on these proteins.

2.2.5. Gene expression modulation

Some studies suggest that DOX down-regulates cardiac muscle-specific proteins such as contractile proteins, mitochondrial proteins, sarcoplasmic reticulum proteins, and others. Suppression of the cardiac muscle gene is associated with abnormal contraction and relaxation observed after DOX treatment [3, 11]. Another study showed that DOX induces depletion of GATA-4, leading commitment of the regulation of sarcomeric proteins expression such as myosin heavy chain and troponin I [61, 62]. In addition, suppression of GATA-4 induced by DOX is also related to the induction of apoptosis, suggesting the essential role of GATA-4 in cell survival [63, 64]. Regarding mitochondrial proteins, there is evidence that the suppression of these proteins after DOX treatment results in disruption of myocardial energy production, thereby causing cardiac dysfunction [3].

On the other hand, DOX induces upregulation of endothelin-1 (ET-1) and its receptors’ expression [65, 66]. An in vivo study has shown that DOX-induced cardiotoxicity was reduced when mice were pretreated with the combined endothelin A/B antagonist (bosentan). In addition, the authors suggest that the reduction of TNF-α and BAX expression, lipid peroxidation, and increased expression of GATA-4 are responsible for cardioprotective effects observed in this study [67]. However, it is unclear if combined blocking of endothelin A/B receptors is necessary or whether selective inhibition of one of the ET-1 receptors is sufficient for the observed cardioprotection. In this context, a recent study evaluated the effects of dual (bosentan) and single endothelin receptor antagonism through sitaxentan (receptor A blocker) or BQ788 (receptor B blocker). The results demonstrated more beneficial effects of cardiac function when both receptors were blocked [66]. Taken together, these data support a substantial role of endothelin-1 signaling as a mediator of DIC.

2.2.6. Apoptosis

DOX can induce apoptosis through different mechanisms, which have been extensively studied in both acute and chronic cardiotoxicity. As mentioned in this chapter, one pathway involves ROS production and oxidative mechanisms and it is accepted that both the extrinsic and intrinsic apoptotic pathways are involved [17]. Increased oxidative stress has been shown to promote apoptosis and antioxidants have been shown to inhibit this process [7]. Oxidative stress also is known to activate apoptosis-signal regulating kinase-1 (ASK1), which activates the c-Jun NH2-terminal kinase (JNK) and p38 MAPK pathways to induce apoptosis [68]. In addition, it is reported that transcription factor NF-κB activated by ROS in DOX-treated neonatal rat cardiomyocytes and myocardium exerts a proapoptotic effect via direct activation of apoptotic genes, including FasL, Fas, c-Myc, and p53 [69–71]. The
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