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Meet the editor

Dr. Wenyong Tan is a clinical oncologist at the Shenzhen Hospital of Southern Medical University. He is currently serving as a professor at both the Southern Medical University and Jinan University. His main areas of interest are lung cancer, breast cancer and head and neck cancer. He has vast experience in the radiotherapy and anticancer drug therapy for malignancies. He has had more than 60 academic papers published in international and Chinese journals. He is devoted to the radiotherapy and anticancer drug therapy of lung, breast, head and neck cancer as well as the anticancer associated cardiovascular toxicities. During the last five years, more than 50 of his academic papers were published.

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Preface

It is my great honor to present the book entitled *Cardiotoxicity*. The authors of this book are researchers in cardiotoxicity and experts in oncology and/or cardiology. This book will improve the development of cardio-oncology as a novel interdisciplinary field.

Nowadays, cancer is becoming the top cause of death in humans and almost more than 80 percent of these patients receive radiotherapy and anticancer drug therapy. As the modalities of cancer treatment grew, both radiotherapy and anticancer agents included cytotoxic, molecularly targeted drugs and some immune checkpoint inhibitors were proved to be associated with acute and long-term cardiovascular side effects that impact the patient's survival outcome and quality of life. Also, some agents such as thiazolidinediones widely used in the treatment of diabetes can cause some cardiotoxicities. Therefore, medical treatment induced cardiotoxicities should be an important concern in the clinical decision. In fact, different agents have their special molecular pathways in the cardiovascular system and the individual strategies should be adapted for each agent. This book covers all these aspects.

This book is divided into three sections with eight chapters. The first section encompasses the biological mechanisms of cytotoxic agents that induce cardiac damage. Most basic and clinical research focuses on the anthracyclines, especially the doxorubicin, associated heart damage and these are discussed in this section. The biological mechanisms of other anticancer drugs such as pyrimidinedione analogs and trastuzumab are also covered. In the second section, the potential mechanism agents used in some other chronic diseases such as thiazolidinediones and cocaine are addressed and some preventable strategies are recommended. In the last section, possible prevention and efficient management strategies were widely discussed. It also covers the novel biomarkers that would be useful to help manage cardiovascular toxicity and develop safer and better tolerated cancer drugs as well as identify the patients with high risk that need to have the individual prevention and treatment strategies.

This book is intended to document the many advancements in the various drugs associated with cardiovascular toxicities, especially the anticancer agents in the oncological field. It also integrates the advancements in oncology and cardiology, which go beyond managing cardiovascular toxicity by the oncologist and/or cardiologist. There are many valuable contributions from the basic researchers and clinicians who are experts in these field. I am grateful to all the contributors for all their efforts in preparation of this book.

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Section 1

Basic Research: Anti-Cancer Agents Associated Cardiotoxicities

Chapter 1

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

Danúbia Silva dos Santos and
Regina Coeli dos Santos Goldenberg

Additional information is available at the end of the chapter

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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, Ca^{2+} 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 to 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

[9]. 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, Ca^{2+}

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 ($O_2^{-\cdot}$) and hydroxyl radicals ($OH^{\cdot}$) as well as other non-radicals such as hydrogen peroxide (H_2O_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 mortality 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 S,S′-[1,3-phenylene-bis(1,2-ethanediyl)]bis-isothiourea (1,3-PB-ITU) and L-N6-(1-iminorthyl)-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 multicomponent 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 (diphenyliodonium and apocynin) on H9c2 cells showed that DOX-induced apoptosis was mitigated, 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

activation of p53 by superoxide and hydrogen peroxide activates Bax genes, causing apoptosis [72, 73]. At the same time, evidence indicates that there is also an increase in the production of proapoptotic proteins as a result of p53 stabilization through increased heat shock protein (Hsp)25 production due to the activation of heat shock factor 1 (HSF-1), which is induced by DOX-dependent oxidative stress. In contrast, several studies suggest that Hsp proteins, such as Hsp27, Hsp10, Hsp20, and Hsp60, are involved in the prevention of DOX-induced apoptosis and myocardial dysfunction [74, 75]. Overexpression of Hsp27 plays a beneficial role in the regulation of oxidative stress responses and maintenance of mitochondrial function [74]. Regarding Hsp10 and Hsp60, overexpression of these proteins is associated with an increase in the post-translational modification of Bcl-2 proteins, which are important for the activation of anti-apoptotic pathways [75]. In addition, it is reported that overexpression of Hsp20 inhibits DOX-triggered cardiac injury, and these beneficial effects appear to be dependent on protein kinase B (also known as Akt) activation [76]. Therefore, more studies should be performed to understand the anti- and/or proapoptotic signaling pathways activated by Hsp proteins and their relationship to DOX.

Recently, an in vitro study using cardiomyocytes derived from human induced pluripotent stem cells (CM-iPSC) showed that DOX significantly upregulated the expression of death receptors (DRs) (TNFR1, Fas, DR4, and DR5) at both protein and mRNA levels. This study also showed that spontaneous apoptosis is exacerbated by death ligands including TNF-related apoptosis inducing ligand (TRAIL) [77]. Another study reported that Toll-like receptor-2 (TLR-2) functions as a novel "death receptor" that employs the apoptotic apparatus such as FADD and caspase 8 without a conventional cytoplasmic death domain. In this study, the authors observed reduction of apoptosis in myocardium after DOX treatment in TLR-2-knock-out mice (TLR-$2^{-/-}$) when compared to wild-type mice [78]. These results demonstrate that the induction of death receptors in cardiomyocytes is probably another mechanism by which DOX causes cardiotoxicity.

DOX is also appearing to influence caspase activity. Using both rat primary cultured cardiomyocytes and rat hearts from an animal model, the study demonstrated that DOX treatment induces apoptosis through the activation of caspase-3 activity [79]. In addition, another study showed that caspase-3 can be activated after Akt and Bad phosphorylation caused by DOX-induced upregulation of Ser/Thr PP1 phosphatase [76]. In support of this view, in TLR-2-knock-out mice, DOX-induced caspase-3 activity was decreased and this effect is a result of inhibition of NF-κB activation and reduction of proinflammatory cytokine [78, 80]. In all, these data demonstrated the need to understand the molecular signaling pathways that mediate DOX-induced cardiomyocyte apoptosis. This knowledge is extremely important for the advancement and development of new approaches for the treatment and/or prevention of DIC.

2.2.7. Other emerging mechanisms: role of microRNAs

Several studies indicate DIC is associated with modulation of microRNAs due to their role in all cardiac functions, including conductance of electrical signals, heart muscle contraction, and growth [81]. It is reported that a group of microRNAs, such as miR-34a, miR-34c,

miR-208b, miR-215, miR-216b, and miR-367 are upregulated in the rat heart when increasing doses of DOX are administered. In this same condition, there is evidence that miR-21, miR-34a, miR-208a, miR-208b, miR-221, miR-222, and miR-320a are upregulated in mice myocardium [81, 82]. On the other hand, other microRNAs including Let-7 g, miR-30a, miR-30c, and miR-30e are downregulated in rat myocardium after DOX treatment, confirming the role of DOX in the modulation of microRNAs [81].

Recently, the effects of DOX on the expression of miR-21 were examined in rat H9C9 cardiomyocytes. This study showed that overexpression of miR-21 attenuated DOX-induced apoptosis, whereas knocking down its expression increased DOX-induced apoptosis. In addition, the authors suggest that miR-21 protects cardiomyocytes by modulating the anti-proliferative factor, B cell translocation gene 2 (BTG2) [83]. Furthermore, the effects of DOX on expression of miR-208a were investigated in Balb/C mice hearts. In this study, DOX significantly upregulated miR-208a, downregulated GATA4, and increased myocyte apoptosis. In contrast, therapeutic silencing of miR-208a recovered GATA4 and BCL-2 and decreased apoptosis [84]. DOX also induced overexpression of miR-146a, which are responsible for downregulating ErB2 receptor tyrosine kinase 4 (ErB4), a key component of neuregulin-1-ErbB signaling, resulting in apoptosis in cardiomyocytes [85].

In turn, a recent study demonstrated that the miR-30 family, which is downregulated by DOX, is involved in the modulating of β-adrenergic and mitochondrial apoptotic pathways. In this study, the authors identify GATA-6 as a mediator of DOX-associated reductions in miR-30 expression. Moreover, they showed that overexpression of miR-30 protects cardiomyocytes from DOX-induced apoptosis [86]. Therefore, these data highlight the importance of modulating microRNA expression as well as providing a novel therapeutic approach to DIC prevention.

2.3. Strategies for heart protection

Since several mechanisms are involved in the development of cardiac toxicity, different strategies are being performed to prevent DOX-induced cardiomyopathy. One of these strategies is the use of dexrazoxane (also known as ICRF-187), an adjunctive agent derivative of ethylenediaminetetraacetic acid (EDTA), which acts as a free radical scavenger. In this case, dexrazoxane is an EDTA-like chelator that interferes with iron-mediated oxygen free radical generation and, consequently, lipid peroxidation [87, 88]. The beneficial effects of dexrazoxane have been demonstrated in murine [89, 90] and canine [91, 92] models. Further, a meta-analysis of six randomized trials that included 1013 adult and pediatric patients demonstrated significantly reduced incidence of heart failure after dexrazoxane treatment, confirming its beneficial effects [93]. In addition, cardioprotective effects have been observed in children with high-risk acute lymphoblastic leukemia (ALL) receiving chronic DOX (10 doses of 30 mg/m^2) [94, 95]. The studies concluded that treatment with dexrazoxane is justified individually when the risk of cardiac dysfunction is expectedly high [87]. Unfortunately, according to the current Food Drug Administration (FDA) approval statement and European Medicines Agency (EMA), the use of this drug as a cardioprotective is limited to women with metastatic breast cancer who have received cumulative doses of 300 mg/m^2 DOX [95, 96].

Another strategy that has been evaluated is the use of angiotensin-converting enzyme (ACE) inhibitors, including enalapril, zofenopril, and lisinopril. ACE inhibitors are commonly used in patients with heart failure as afterload-reducing agents. In addition to their features as an effective ACE inhibitor, these drugs act as antioxidant and, thus, may contribute to prevent cardiac toxicity [97]. In support of this view, recent preclinical study demonstrated that administration of enalapril attenuated DOX-induced cardiac dysfunction via preservation of mitochondrial respiratory efficiency and reduction in DOX-associated free radical generation [98]. Unluckily, in long-term survivors of childhood cancer treated with DOX, the beneficial effect of enalapril-induced improvement in left ventricle structure and function was lost after 6 or 10 years. It is important to mention that ACE inhibitors have adverse side effects and, therefore, the choice of this drug as a cardioprotective agent during cancer treatment should be carefully evaluated [99].

Another antioxidant that has already been tested against DOX-induced cardiomyopathy is vitamin E. A study has shown that vitamin E only prevents the acute effects of DOX cardiotoxicity in mice [100]. Several other antioxidants also have been tested with limited success, including vitamin C, reduced glutathione, selenorganic compound PZ51, oleanolic and ursolic acids, and ambroxol [101–105]. On the other hand, probucol, a lipid-lowering agent and potent antioxidant, provided complete protection against DOX-induced cardiomyopathy and heart failure in animal experiments without interfering with the antitumor properties of this antibiotic [106, 107]. In this case, it is extremely important that clinical trials using DOX therapy in combination with probucol are performed to determine a new preventive cardiotoxicity strategy. This approach was recently tested using a β-adrenergic receptor blocker and also an antioxidant agent called carvedilol. As result, this drug protected systolic functions of the left ventricle due to reducing DIC [108]. Therefore, the use of carvedilol may become a promising strategy to improve DIC. However, more studies are needed to assess whether the beneficial effect observed on cardiac function is preserved over the years.

In addition to the antioxidant agents, accumulating evidence indicates that cardiac α1-adrenergic receptors (α1-ARs) protect cardiomyocytes from DIC. In particular, the stimulation of α1-AR-specific agonists phenylephrine (PE) and dabuzalgron have been shown to reduce apoptosis, interstitial fibrosis, and myocardial dysfunction caused by DOX. This protective effect is associated, at least in part, with the expression of anti-apoptotic proteins of the Bcl2 family and preservation of mitochondrial function [64, 109]. Thus, further studies will be needed to elucidate the full mechanisms responsible for the cardioprotective effects observed up to now.

2.4. Breakthroughs and challenges in basic and clinical research

Although therapeutic strategies to prevent cardiomyopathy have been proposed for more than four decades, it is important to highlight that there is still no specific treatment for total recovery of the myocardial injury caused by DOX. In this case, cardiac transplantation remains a vital option for patients with end-stage heart failure due to DOX-induced cardiomyopathy [3]. However, the major problem is the long time of wait in the queue of transplant due to low donor/acceptor ratio. Statistical data show that 10–20% of the patients in the waiting queue come to death annually [110].

In this scenario, in which therapeutic options for DOX-induced cardiomyopathy are insufficient, a newly emerging strategy is cell therapy. The principle of cell therapy is to restore the function of an organ or tissue by transplanting new cells [111]. In this context, a study has shown that transplanted mouse embryonic stem cell (ESC) in DOX-induced cardiomyopathy mice model attenuated various pathological mechanisms such as: (1) cardiomyocyte apoptosis due to inhibition of phosphoinositol-3-kinase (PI3K)/Akt and ERK pathway; (2) cardiac fibrosis; (3) cytoplasmic vacuolization; and (4) myofibrillar loss [112]. Although beneficial effects have been observed in this study, the teratogenic potential of these cells represents serious limitation to their use [113, 114]. In fact, experimental models of myocardial infarction have demonstrated the formation of teratomas after the transplantation of undifferentiated ESC [115, 116]. Thus, to overcome this limitation, several studies suggest the use of already differentiated ESC in cardiomyocytes [116, 117].

Considering that DOX causes cardiomyocyte death through the activation of different molecular and pathophysiological mechanisms, this therapeutic approach seems to be promissory for future application against DIC. In support of this view, recently, our research group has shown that the transplant of cardiomyocytes derived from mouse ESC (CM-mESC) improved cardiac function and electrical activity of the mice hearts with DIC, as well as reduced the percentage of cardiomyocyte apoptosis [118].

For clinical research, cardiomyocytes derived from human induced pluripotent stem cells (CM-iPSC) are potential cell sources for cardiomyocyte transplantation therapy. The generation of induced pluripotent stem cells (iPSC) from human somatic cells through overexpression of four transcription factors (OCT4, SOX2, c-Myc, and KLF4) represented a scientific milestone opening new perspectives for treatment of heart diseases. In addition to showing the same characteristics of ECS and, thus, the ability to differentiate into cardiomyocytes, these cells are not associated with immune rejection [119, 120].

Currently, the great challenge for the scientific community is the development of pro-maturation strategies to obtain human adult cardiomyocytes in vitro, with ventricular-like phenotype based on action potential, genetic, morphological, and metabolic characteristics [121]. Once maturation is achieved, CM-iPSC are a viable option as an autologous cell source for cardiac repair and a powerful tool for treatment of cardiovascular diseases, including DIC.

3. Conclusion

DIC is an important public health concern given the fact that this disease may not to be detected for many years and remains a life-long threat. Mechanisms contributing to the development of cardiomyopathy involve (1) free radical generation; (2) alteration in iron metabolism through iron-DOX complex formation or interference in the proteins' activity that transport and bind intracellular iron; (3) increased calcium intracellular concentrations; (4) disarray and loss of myofilaments of the sarcomere; (5) gene expression modulation; (6) activation of apoptosis by different signaling pathways; and (7) modulating microRNA expression. Based on these mechanisms, a variety of strategies to prevent cardiotoxicity have been tried, including the use of iron-chelating antioxidants and adrenergic receptor agonists. However, so far, the ability of these treatments to protect the heart from DOX-induced damage has been limited. Since DOX causes cardiomyocyte death, one recent approach that has shown promise is the transplant of

CM-iPSC. In this context, the scientific community has been engaged in the establishment of pro-maturation protocols to obtain adult human cardiomyocytes in vitro. Once this challenge has been overcome, we believe that cell therapy with CM-iPSC may be a promising strategy for the development of effective therapy against DIC.

Conflict of interest

The authors declare that they have no conflict of interest.

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Chapter 2

Doxorubicin Cardiotoxicity: Multiple Targets and Translational Perspectives

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Additional information is available at the end of the chapter

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Abstract

Anthracycline cardiotoxicity remains a serious problem in pediatric and adult cancer survivors. This chapter discusses the involvement of multiple cardiac cell types in the pathogenesis of the onset and progression of doxorubicin cardiotoxicity. In addition to cardiomyocytes, considered the classical cellular target, the role of cardiac fibroblasts and vascular cells together with progenitor cells of cardiac and extra-cardiac origin is addressed with a focus on oxidative stress, DNA damage, senescence, cell death, and molecular signals involved in cellular injury and response. Current strategies for primary and secondary prevention aiming at contrasting the onset of early and late doxorubicin-induced toxic events do not completely resolve the growing clinical problem. Thus, there is the necessity to understand cellular processes that operate within and beyond cardiomyocyte, to develop more effective tools for the prevention and treatment of progressive cardiomyopathy in otherwise successfully treated oncologic patients.

Keywords: anthracycline cardiomyopathy, cellular targets, molecular mechanisms, progenitor cells, cardioprotective strategies

1. Introduction

Cardiotoxicity is one of the most serious consequences of cancer therapy. Over the last decades, this complication has reached alarming dimensions due to aging of the population, epidemics of chronic comorbidities, and improvement in patient survival through to the increasing access to innovative diagnostic and therapeutic approaches. Cardiovascular death may represent a greater threat of mortality than cancer itself, and its risk, following cancer treatment,

may be higher than the actual risk of tumor recurrence. Indeed, cardiovascular death is the leading cause of death in female breast cancer patients, and exceeds that of cancer recurrence in childhood cancer survivors [1]. The growing awareness of the anticancer therapies-related cardiovascular risk, which can influence the appropriate management of patients, stimulates continuous effort in basic and clinical research.

Anthracyclines, such as doxorubicin (DOX), discovered in Italy, have been widely used in the treatment of various malignancies since the 1960s, although they can cause ventricular dysfunction and cardiomyopathy that were recognized right after their introduction to clinical practice [2, 3]. Contrary to public awareness, the scale of the anthracycline cardiotoxicity issue is by no means minor. A nine-year follow-up of patients treated with anthracyclines showed, respectively, 17.9 and 6.3% of subclinical and overt cardiotoxicity [4]. The cardiotoxic effect may be either reversible or irreversible. The former occurs during or shortly after drug infusion, is dose independent, and manifests as a transient decline in myocardial contractility with various electrocardiogram abnormalities. The latter can be classically divided into early and late onset toxicity. The early onset effects appear several weeks or months after the last dose of anthracycline. This is the most frequent and clinically relevant form of cardiotoxicity, usually presenting as a dilated cardiomyopathy leading to heart failure (HF). The late onset toxicity may appear with the occurrence of HF symptoms, years or even decades after the end of chemotherapy, especially in childhood cancer survivors [5]. The prognosis in anthracycline-related HF is relatively poor. However, the increasing attention to early detection of ventricular dysfunction and appropriate management can significantly improve the outcome.

Although the introduction of new synthetic and biological molecules has improved the course of many cancers, anthracycline chemotherapy regimens still have a prominent role in clinical protocols. Long-term survival creates a growing population of cancer patients who will be, for years to come, at risk of cardiovascular morbidity and mortality due to anthracycline chemotherapy.

2. Molecular and cellular mechanisms

Anthracycline-induced cardiotoxicity has stimulated substantial interest of basic and clinical researchers over the years, although the pathogenetic mechanisms have not been completely clarified.

2.1. Cell loss

It is well known that cardiotoxicity induced by anthracycline involves the activation of molecular pathways triggering the loss of cardiomyocytes, and that these cell death mechanisms, either apoptotic or necrotic, may occur in an acute phase, soon after anthracycline exposure. Experimental studies have shown anthracycline to induce cell death in a concentration-dependent fashion, with apoptosis occurring at lower and necrosis at higher concentrations of the drug [6, 7]. Specifically, DOX activates mitogen-activated protein kinases (MAPK), such as p38 and c-Jun N-terminal kinase (JNK), which leads to apoptosis via oxidative stress, calcium handling impairment, mitochondrial swelling, and caspase activation [8]. Although

autophagy is referred to as a physiologic process, abnormal autophagy may be involved in various diseases, taking part in the pathogenesis of HF including anthracycline-induced cardiomyopathy [9].

2.2. The redox element

Traditionally, the main mechanism accounting for cardiotoxic potential of anthracyclines has been attributed to the excessive production of reactive oxygen and nitrogen species (ROS and RNS) [10]. This overproduction is facilitated by permissive conditions due to low antioxidant capacity, in terms of ROS-scavenging enzyme synthesis, possessed by a cardiomyocyte. Antioxidant enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidase, cytochrome P450, and glutathione transferases are less expressed in the heart compared to other organs. In addition, the level of antioxidants is further lowered by DOX that decreases the expression of catalase and Cu/Zn-SOD [11–13].

The reduction of the quinone moiety in ring C transforms DOX to semiquinone, which generates $O_2^{\bullet -}$ (when reacting with oxygen) that in turn is neutralized into low-toxic hydrogen peroxide (H_2O_2) by superoxide dismutase. Alternatively, H_2O_2 and $O_2^{\bullet -}$ react with one another to generate highly reactive $OH^{\bullet}$ according to the iron-catalyzed Haber-Weiss reaction [14].

The subcellular compartment where most of ROS are produced is the mitochondrion, where DOX, being accumulated for its high affinity to cardiolipin, a phospholipid located in the mitochondrial inner membrane, determines deleterious effects by producing ROS and disrupting the electron-transport chain. In addition to mitochondrial dysfunction, high levels of ROS and RNS, triggering cytotoxic signaling, lead to DNA and protein oxidation, and impairment of intracellular calcium homeostasis. Upregulation of Mn-SOD, situated in the mitochondrial matrix and serving as another ROS scavenger, prevents the accumulation of free radicals in mitochondria [15]. DOX impairs the correct synthesis also of this antioxidant enzyme [16].

At the same time, lipid peroxidation is induced by DOX through a non-enzymatic reaction that reduces Fe^{3+} and forms DOX-Fe^{2+} free radical complexes. As a main consequence, lipid peroxidation alters the membrane structure and permeability influencing on cell signaling and function. To control the process of free radical generation, the iron is sequestered by storage and transport proteins. By counterpart, DOX impairs iron homeostasis and determines iron accumulation in mitochondria, leading to cardiac cell damage [17].

Numerous enzyme systems are accountable for ROS production, including reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOXs), xanthine oxidases, uncoupled nitric oxide (NO) synthase (NOS), and enzyme complexes of peroxisomes. NOXs are multi-subunit transmembrane enzymes that utilize NADPH as an electron donor to reduce oxygen to $O_2^{\bullet -}$ and H_2O_2. NOX2 and NOX4 isoforms are predominantly expressed in the heart where they contribute to enhancement in oxidative stress. Growing evidence has demonstrated an increased and persistent activation of these enzymes in response to DOX exposure [10].

NOSs are another source of DOX-dependent ROS. DOX, by binding to endothelial NOS (eNOS), interferes with NO generation in favor of superoxide formation. ROS, reacting with NO to generate RNS, boost the generation of oxidants that, in turn, force the uncoupling of

eNOS, altering the enzyme function to produce more $O_2^{\bullet-}$ and less NO. The formation of peroxynitrite leads to mitochondrial oxidative damage with a consequent apoptosis and/or necrosis [18].

Nuclear factor erythroid 2-related factor 2 (Nrf2), a basic leucine zipper protein, is involved in the expression of diverse antioxidant proteins and plays a crucial role in DOX-induced cardiomyopathy. Whereas deficiency of Nrf2 aggravates cardiotoxicity and cardiac function, overexpression of Nrf2 has a protective effect on the myocardium. The exact mechanism remains elusive, but it seems that Nrf2 mediates the balance between oxidative stress and autophagy [19].

2.3. Topoisomerases

DOX-dependent oxidative stress is not the only phenomenon at the base of anthracycline cardiotoxicity. Recent findings have attributed DOX binding to topoisomerase II, a leading mechanism driving cardiac abnormalities. DOX anticancer activity is partly due to the ability to form, in cancer cells, ternary complexes with topoisomerase IIα and DNA, causing DNA double-strand breaks and cell cycle arrest, and eventually activating death processes. However, other topoisomerase isoforms are also targeted by DOX. In contrast to α isoform (mainly expressed in rapidly dividing malignant and non-malignant cells), topoisomerase IIβ is highly expressed by quiescent cells (i.e. adult cardiomyocytes), and its inhibition mediates DOX-driven cardiotoxic effects through DNA damage and defective mitochondrial biogenesis [20, 21]. According to this scenario, the interaction between DOX and topoisomerase IIβ accounts as the initial event of cardiotoxicity whereas oxidative stress must be considered as a consequential step. Being either originating or downstream event, oxidative stress plays a fundamental role in anthracycline cardiotoxicity and its modulation is still subjected to experimental and clinical investigation.

2.4. Myocardial senescence

Induction of long-term cardiotoxicity is attributable to molecular mechanisms other than those of apoptosis and necrosis. Premature myocardial senescence has been recognized as an essential phenomenon in the development of HF [22]. DOX affects several molecular and cellular events, such as activation of p53, $p21^{Cip1}$, and $p16^{INK4a}$, leading to the cells' replicative stress and impaired cellular functions [23]. Importantly, terminally differentiated cells, including adult cardiomyocytes, with a senescent phenotype, are still metabolically active and capable of synthesizing pools of cytokines, growth factors, and regulatory enzymes known as senescence-associated secretory phenotype or senescence messaging secretome that affects the surrounding microenvironment [24].

2.5. Ion homeostasis

DOX cardiotoxicity is associated with dysregulation of sodium and calcium homeostasis that develops after ROS generation. DOX alters the expression of many calcium exchange regulating proteins such as sodium-calcium exchanger, ryanodine receptor, sarco/endoplasmic reticulum adenosine triphosphate (ATP) hydrolase 2a, and phospholamban. From the functional

point of view, changes in the expression of sodium/calcium-regulating genes impair both systolic and diastolic performance. Notably, the activation of calcium/calmodulin-dependent protein kinase II was found to be a key aspect leading to the changes in genes involved in ion signaling in DOX cardiomyopathy [25, 26].

2.6. Ultrastructural changes

An additional effect of anthracyclines on cardiomyocytes implicates the disruption of sarcomeric structure. Studies conducted on patients and rodents exposed to anthracyclines have demonstrated ultrastructural changes, such as loss and disarray of sarcomeric myofibrils, dilation of sarcoplasmic reticulum, swelling of mitochondria, and cytoplasmic vacuolization. The integrity of sarcomere is essential to myocyte dynamics, and deficit in assembly or organization of cardiac sarcomeres ultimately leads to impaired cardiac function [27].

2.7. Cell energetics

Reduced contractility can be also linked to the disturbed myocardial energetics. Negative inotropic effect of anthracyclines as well as the irregular energy-dependent phase of cell relaxation can be linked to intracellular abnormalities. DOX decreases ATP and phosphocreatine levels, thus decreasing cardiac energetic reserve in terms of the availability of high-energy phosphates. Interestingly, the reduced phosphocreatine/ATP ratio was observed in patients without clinical evidence of cardiomyopathy and even years after chemotherapy [28]. Along with the decreased energy production, DOX impairs energy sensing and high-energy phosphate transfer, affecting creatine kinase isoenzymes. Also, in addition to abnormal fatty acids oxidation, DOX limits the compensative response in terms of glucose utilization [29]. Overall, deficit in high-energy phosphate metabolism and poor performance of compensatory and regulatory circuits significantly contribute to the onset and progression of anthracycline cardiomyopathy.

2.8. Non-coding RNAs

Biological importance of regulatory function of RNA is reflected in the fact that more than 98% of the transcriptional product of the genome is a non-coding RNA. In a recent study, global transcriptional profiling has identified DOX-induced changes in the levels of several cardiac RNA-binding proteins (RBPs), including downregulation of Quaking isoform 5 (Qki5). RBPs control the function of coding and non-coding RNAs. Qki5 was shown to regulate the formation of circular RNAs and has protective role against DOX-induced cardiomyocyte death and cardiac dysfunction. Several circular RNAs, controlled by overexpression of Qki5, were also downregulated in response to DOX. Interestingly, inhibition of titin gene-derived circular RNA increased the susceptibility of cardiomyocytes to DOX [30].

3. Cardioprotective strategies

The uncertainties regarding the management of anthracycline cardiotoxicity have evidenced the necessity to develop a multidisciplinary modus operandi that intertwines cardiologists'

and oncologists' expertise for the best management of cardiovascular outcomes in patients undergoing anticancer therapy. Prevention of cardiotoxicity begins before starting anticancer therapy, with the evaluation of the cardiovascular risk profile and the adoption of strategies to reduce such a risk (blood pressure and blood glucose control, smoking cessation, and cholesterol reduction).

3.1. Dose reduction

The DOX cumulative dose is the most important risk factor for the development of cardiotoxicity. The estimation of patients with DOX-related HF rises from 5% at a cumulative dose of 400 mg/m^2, to 16% (500 mg/m^2), 26% (550 mg/m^2), and 48% (700 mg/m^2) [31]. Therefore, clinical protocols recommend not exceeding 400–450 mg/m^2 cumulative dose. It is worth noting that late onset cardiac abnormalities have been observed in patients treated with DOX cumulative dose well below the "safety threshold," suggesting that there may be no safe dose of anthracyclines [32]. In children exposed to DOX dose lower than 100 mg/m^2, cardiac abnormalities were detected in 30% of survivors after several years [33].

3.2. Pharmacokinetic approach and analogs

Alternative or additional strategies of primary intervention are used to reduce or prevent deleterious effects that anthracyclines have on the heart. Standard approaches are based on the differences in pharmacokinetic aspects of antitumor activity and cardiotoxicity. Whereas anthracycline antitumor efficacy corresponds to total exposure, cardiotoxicity correlates with the peak plasma level. Thus, these methods consist in changing administration schedule by replacing bolus with slow infusion and switching from conventional to liposomal formulations. Continuous slow infusion lowers peak concentration, thus reducing cardiotoxicity, but retains anticancer activity. However, cardioprotective benefits have been proved in limited therapeutic protocols, and elevated costs of longer hospitalization and risk of infections represent additional causes that have limited its use [14]. Liposomal encapsulation enables a preferential crossing of irregular vasculature (tumor tissue) instead of less permeable vessels of healthy tissues, thus modifying tissue distribution of DOX. Uncoated or pegylated liposomal anthracyclines have proved to be as effective as conventional formulations but with less toxic effects. Nonetheless, liposomal anthracyclines have been investigated in relatively few randomized trials so that their use is approved for only limited clinical indications, such as metastatic breast cancer, ovarian cancer, multiple myeloma, and acquired immune deficiency syndrome-related Kaposi sarcoma [34]. The costs of such preparation represent also a limiting factor. DOX analogs (i.e. epirubicin and idarubicin) have been introduced into clinical practice in place of DOX with the scope of reducing cardiotoxicity. These molecules are effectively less cardiotoxic than DOX, but being less active as well, it is necessary to augment the dose to maintain the antitumor activity equivalent to DOX, thus increasing the risk of HF, particularly in patients with defined risk factors [35].

3.3. Antioxidants

The role of ROS in the pathogenesis of anthracycline-related cardiotoxicity and HF has provided the basis for testing the coadministration of synthetic drugs or natural compounds with

antioxidant properties to counteract the development of cardiotoxicity. Early studies assessing the efficacy of dietary supplements such as vitamin A, vitamin E, coenzyme Q10, and other compounds known to prevent oxidative damage have produced disappointing results. Although some of these molecules (of which there are data on the tumor response rate) do not disempower the antineoplastic efficacy of the anthracycline, the benefits on myocardial function were modest at most [36].

Dexrazoxane is the only approved drug used in clinical settings as cardioprotective agent in pediatric and adult patients exposed to DOX cumulative dose known to induce HF. Dexrazoxane interferes with DOX-dependent redox reactions, and decreases ROS production and tissue damage by chelating iron before it catalyzes the conversion of superoxide anion ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) into highly reactive hydroxyl radicals ($OH^{\bullet}$) [37]. Recently, it has been demonstrated that dexrazoxane can compete for the ATP-binding site of topoisomerase IIβ, thus precluding the formation of anthracycline-DNA-topoisomerase IIβ complex and thus preventing DNA double-strand breaks and cardiomyocyte death. This new mechanism of cardioprotection may explain why it has succeeded while other antioxidants have not [38]. However, the clinical use of dexrazoxane has been limited following few reports of its possible interference with antitumor activity of anthracyclines, and the potential risk of a second malignancy in pediatric patients. Although numerous evidence has denied these concerns, the regulatory agencies have maintained the limitation of the use of dexrazoxane in restricted clinical conditions [39].

3.4. Cardiovascular drugs

Additional options include the use cardiovascular prophylaxis with β-blockers, angiotensin-converting enzyme (ACE) inhibitors, angiotensin receptor antagonists (ARBs), and statins. Two β-blockers, carvedilol and nebivolol, have shown to be protective against DOX-induced cardiotoxicity. The mechanisms have not been fully elucidated, but it seems clear that the protective effects lie beyond β-adrenergic antagonism, as not all β-blockers are effective. According to several studies, the antioxidant activity of carvedilol prevents lipid peroxidation and endogenous scavenger breakdown, while the inhibition of peroxynitrite generation and NOS uncoupling may be underlying protective mechanisms of nebivolol [40, 41]. Numerous evidence suggests that a key role in the development and progression of anthracycline-induced cardiotoxicity is played by the renin-angiotensin-aldosterone system, highlighting a potential benefit from the use of ACE inhibitors and ARBs. Preclinical and clinical studies have demonstrated that the inhibition of the renin-angiotensin-aldosterone system can mitigate cardiac dysfunction induced by anthracyclines [1]. Of note, the association of a β-blocker (carvedilol) with the ACE-inhibitor (enalapril) has provided the most effective response toward the amelioration of anthracycline-caused myocardial functional deficit, when either administered preventively, without any signs of systolic dysfunction, or promptly given after the detection of ejection fraction fall [5]. Also, statins have shown to be effective in the prevention of cardiotoxicity by reducing the risk of HF and cardiac-related mortality, due to their pleiotropic effects. The antioxidative property and the capacity of reducing cardiac inflammation may be the main mechanisms that support the role of statins as cardioprotective agents in this scenario [42].

3.5. Non-pharmacological approach

Lifestyle changes and exercise represent non-pharmacological measures to counter anthracycline cardiotoxicity. Clearly, exercise positively modulates several cardiovascular risk factors that need to be considered when assessing the risk of cardiotoxic event. But there is also a possible mechanistic link as exercise diminishes pro-apoptotic signals, improves calcium handling and myocardial energetics, and reduces ROS production. However, more data are needed to assign a role of exercise in the prevention and treatment of anthracycline cardiotoxicity [43, 44].

Despite all the progress made, more and more researches and prospective studies are needed to develop clinical practice guidelines that may direct the specialists to choose an interventional strategy (prophylactic and/or therapeutic) tailored to the characteristics of cancer patients.

4. Beyond a cardiomyocyte

Decades of research to elucidate anthracycline cardiotoxicity have exclusively focused on cardiomyocytes. However, they account for less than one-third of the total number of cells within the heart, whose proper function depends on a complex cellular network. Therefore, attention may be warranted to other cardiac and non-cardiac cell populations such as progenitor cells, vascular cells, and fibroblasts that have been suggested as additional targets in the development as well as management of anthracycline toxicity [45–48].

The following sections will expose the research on representative populations of cardiac cells (non-cardiomyocytes), whose depressed function induced by the anthracycline provides additional insights to further elucidate the complexity of cardiotoxicity. This expanding knowledge may also serve as the basis for innovative preventive and therapeutic interventions.

4.1. Cardiac progenitor cells

It has been repeatedly demonstrated that the myocardium contains an endogenous reservoir of cells with the ability to repopulate the damaged tissue. One of the best characterized populations of primitive cells is represented by cardiac progenitor cells (CPCs). They are cells expressing the stem cell marker (c-kit), residing in the myocardium, where they contribute to tissue homeostasis/repair [49–57].

4.1.1. CPC senescence and functional deficit

The increasing number of researches showing that DOX has CPCs as a cell target, with negative effects on their biological function, suggests a novel mechanism of cardiotoxicity [58–64]. In a rodent model of anthracycline cardiomyopathy, DOX increased ROS-dependent DNA damage, cellular senescence, cell cycle arrest, and apoptosis, affecting CPC viability, growth, and functional activities. In the failing heart, the loss of CPC pool interfered with mechanisms that account for the restoration of structural integrity and functional performance [58].

Supporting evidence has come from another study analyzing the heart of oncologic patients, who died of HF after being treated with anthracyclines. In comparison to age-matched patients who died of non-cardiovascular causes, the myocardium of DOX-treated patients showed a higher number of CPCs positive for the phosphorylated form of histone H_2AX and $p16^{INK4a}$, indicating increased levels of DNA damage and cellular senescence, respectively. In addition, *in vitro* exposure to DOX of human CPCs displayed the activation of senescent and apoptotic pathways, corroborating the hypothesis that a CPC dysfunction may be responsible for a higher susceptibility of the myocardium to the increased workload and injury [59]. Indeed, DOX-exposed human CPCs were no longer able to promote structural and functional recovery when injected in the heart of animals with DOX cardiomyopathy, confirming the ineffectiveness of DOX-treated CPCs in fulfilling their functional role in the diseased myocardium [60]. A recent study conducted on human CPCs confirmed the role played by senescence and apoptosis as main mechanisms activated by DOX. The rate of senescent CPCs, increased after DOX, was significantly reduced by the pretreatment with a human amniotic fluid stem cell secretome [62]. Similarly, the *in vitro* treatment with resveratrol, a sirtuin 1 activator with intrinsic antioxidant properties, was able to prevent DOX-induced senescence and growth arrest of CPCs by decreasing accumulation of intracellular ROS and enhancing antioxidant enzymatic defense. The uselessness of DOX-exposed CPCs to guarantee supportive role in the diseased myocardium has been demonstrated with the lack of any structural and functional recovery after the administration of *in vitro* DOX-treated CPCs in the heart of animals with anthracycline cardiomyopathy. On the other hand, priming CPCs with resveratrol partly restored their capacity to counteract the progressive ventricular dysfunction induced by DOX [60].

The definition of the role of growth factors as major determinants of CPC function has been addressed by studying the impact of insulin-like growth factor-1 (IGF-1) and hepatocyte growth factor (HGF) signaling. The activation of IGF-1 receptor promotes proliferative and anti-apoptotic effects, while the stimulation of HGF receptor (c-Met) supports cell migration toward injury areas [65–68]. DOX showed reduced expression of IGF-1 receptor and c-Met by CPCs, revealing the impairment of pro-survival signaling and deficiency of migratory capability, thus aggravating the inadequate response of cardiac repair signaling in the injured heart [59].

4.1.2. CPCs and late cardiotoxicity

Despite decades of studying the molecular mechanisms at the base of late onset DOX cardiotoxicity, no general agreement has yet been reached. It is likely that even the exposure to DOX at a dose that does not determine symptomatic manifestations of cardiotoxicity, makes the heart more susceptible to successive injuries, with progenitor cell dysfunction and/or impaired angiogenesis. To address this issue, experiments were done in juvenile mice that were exposed to a cumulative dose of DOX that did not induce acute cardiotoxicity and, once the animals reached an adult age, they were subjected to myocardial infarction. DOX-exposed mice were more sensitive to myocardial infarction, with a greater extent of infarct size, a lower blood vessel formation in the infarct border zone, and more fibrotic tissue accumulation, compared to infarcted mice that were not exposed to DOX as pups. Moreover, DOX reduced the number of CPCs in the myocardium, and significantly overexpressed the cell cycle inhibitor $p16^{INK4a}$, suggesting the involvement of cellular senescence of cardiac progenitors as one of

the mechanisms responsible for the higher vulnerability of the heart. DOX impaired CPC functional competence by inhibiting cell growth and differentiation capacities *in vitro* [63]. According to this evidence, CPCs "poisoned" by DOX fail to migrate toward the site of injury with a consequent defect in myocardial repair.

The key impact of senescence and time-dependent evolution of molecular effects on CPCs was confirmed in studies on human cells. Human CPCs were briefly exposed to DOX and then cultured in a DOX-free medium. Early after exposure, DOX significantly increased apoptosis along with the expression of proteins involved in DNA damage response, such as ataxia telangiectasia mutated (ATM) kinase and p53. Both cell death and expression of p53 and ATM proteins returned to baseline after DOX washout, while at the same time, the increasing fraction of $p16^{INK4a}$-positive senescent cells indicated that CPCs entered the irreversible phase of growth arrest [59]. These data support the hypothesis that an early toxic event can justify a delayed response that transforms a latent and asymptomatic myopathy into overt HF.

4.1.3. CPCs and non-coding RNAs

MicroRNAs (miRs) are emerging as regulatory factors in cardiovascular physiology and pathology by contributing in the modulation of biological processes such as response to oxidative stress and cellular damage [69]. miRs have been associated with the regulation of myocardial cell proliferation and differentiation [70], and among others, the miR-34 family, particularly miR-34a, is expressed in the heart and is associated with DNA damage, and pro-senescent and pro-apoptotic mechanisms [71, 72]. Recent study has demonstrated the increased expression of miR-34a in rat CPCs after exposure with DOX. miR-34a increased senescent and apoptotic signaling by activating $p16^{INK4a}$ and p53, and when released by DOX-treated CPCs, affected viability and function of other cardiac cells (cardiomyocytes, fibroblasts, and endothelial cells) [61]. This result indicates a paracrine mechanism, already known in other cardiovascular diseases [73]. However, the implications of miR-34a modulation in oncologic patients, as well as the hypothetical role as biomarker of myocardial damage need to be further assessed.

4.2. Vascular cells

In cancer biology, angiogenesis has a central role, given the necessity to form new blood vessels to support the growth of tumor mass. For this reason, continuous researches have pointed the attention on the effects exerted by cytotoxic drugs upon the vascular system [74]. Vascular damage can be directed on non-tumor tissues as well, supporting the concept that endothelial toxicity may be an additional aspect of antineoplastic therapies, including anthracycline-induced cardiovascular alterations [75, 76]. Studies on endothelial cells demonstrated increases in oxidative stress and DNA damage after anthracycline exposure, with impaired endothelial function and disruption of nitric oxide/superoxide balance [77–79]. In endothelial cells, DOX-induced increase in apoptotic rate was associated to elevated intracellular calcium levels and enhanced transcription of eNOS, suggesting a role for eNOS in DOX-mediated endothelial cell death [77]. Moreover, it has been shown that DOX directly binds to eNOS. This determines the reduction of DOX to the semiquinone radical and a consequent increase in superoxide formation and decrease in NO formation [80]. In this way,

a new concept of cardiac microvascular injury as a potential primary event contributing to anthracycline cardiotoxicity has emerged. DOX may affect the function of cardiac endothelial cell barrier by inhibiting the formation of tight junctions and determining an augmented vascular permeability [81].

Besides a direct effect on skeletal muscle microcirculation [82], the treatment with DOX has also proven to directly affect the biology of vascular smooth muscle cells (SMCs). Exposure to DOX produced DNA damage, generation of ROS, and increased senescence-associated β-galactosidase (SA-β-gal) activity in SMCs, which underwent premature senescence and cell cycle arrest [83]. Toxicity of DOX on vascular system was confirmed in organ culture by the detection of a lower capacity of vessels to relax. The involvement of oxidative stress was evidenced by a partial restoration of contractility in the presence of superoxide dismutase [84, 85]. Moreover, DOX induced an upregulation of endothelin-1, the potent vasoconstrictor and pro-inflammatory peptide, whose effects are noticeable during development of cardiovascular disease. Experimentally, endothelin receptor antagonism ameliorated DOX-induced cardiac dysfunction [86]. These data further support the view of anthracycline cardiotoxicity as a multicellular effects-driven process. This can stimulate future studies aiming at characterizing mechanisms of vascular toxicity and helping to design strategies to prevent or minimize the negative impact of DOX on vascular cell function.

4.3. Cardiac fibroblasts

Cardiac fibroblasts have been underrated for a long time. These cells, however, are essential for maintaining cardiac function and take a vital part in cardiac remodeling during pathological conditions. Initiation and maintenance of fibrogenic response is regulated by a complex interaction of growth factors and cytokines. Transforming growth factor-β (TGF-β) signaling is considered the most potent activator of fibroblasts that differentiate into myofibroblasts, cells capable to release great amounts of extracellular matrix components, such as collagen and fibronectin [87–91]. Myocardial fibrosis is a common feature of a broad variety of cardiovascular pathologies including anthracycline cardiomyopathy [87]. Indeed, treatment with DOX promoted phenotypic transformation of cardiac fibroblasts into myofibroblasts both *in vivo* and *in vitro* [92]. As signaling molecules, ROS, through NADPH oxidases, are implicated in the amplification of TGF-β-related pathways that promote fibroblast differentiation, and ultimately cardiac fibrosis [93]. In a rat model of DOX cardiomyopathy, increase in oxidative stress was associated with the upregulation of TGF-β, connective tissue growth factor, and SMAD3 determining adverse matrix remodeling with accumulation of collagen type I.

Pro-fibrotic phenotype and premature myocardial aging coalesce in anthracycline cardiotoxicity. In cardiovascular diseases, senescence is a well-recognized process that contributes to inflammation and myocardial fibrosis and stimulates the production of several factors including IL-6, IL-8, TGF-β, and tumor necrosis factor α (TNFα) [24, 94, 95]. TNFα may have a relevant role as cardiotoxic molecule, since the upregulation of its receptor was detected after DOX exposure in apoptotic myocardial cells [96, 97]. In a recent study, cardiac fibroblasts exposed to DOX prematurely acquired a senescent phenotype, as shown by the increases in SA-β-gal activity and the expression of senescence markers $p16^{INK4a}$ and $p21^{Cip1}$ [98]. As in other cell types, DOX induces DNA damage-response also in fibroblasts. The activation of the stress

sensor ATM kinase catalyzes the phosphorylation of p53, and determines increased expression levels of p53 and $p21^{Cip1}$. Cardiac fibroblasts have been even proposed as the principal cells that mediate cardiotoxic effects of DOX. It has been shown that ATM, activated mainly in cardiac fibroblasts, stimulates the release of Fas ligand, thus promoting DOX-induced cardiomyocyte apoptosis [99]. In addition to the activation of DNA damage-response cascade, in pulmonary fibroblasts, DOX produces a prompt reduction in acetyl-CoA carboxylase 1 expression, the enzyme that catalyzes the rate-limiting step in fatty-acid synthesis [100]. Overall, these processes can regulate the equilibrium of the myocardium and contribute to the switch to a pro-fibrotic profile. Further studies will need to determine the relative contribution of cardiac fibroblasts in the pathophysiology of anthracycline cardiomyopathy and establish the significance of fibroblast-cardiomyocyte cross talk in drug-induced cardiotoxicity.

4.4. Mesenchymal stem cells

To broaden the perspective of the pathophysiology of anthracycline cardiotoxicity, in addition to intracardiac cells (both mature and primitive), extracardiac undifferentiated cells can also be taken into consideration. Mesenchymal stem cells (MSCs) are identified as a fibroblast-like population, originally isolated from bone marrow but present in other tissues as skeletal muscle, adipose tissue, cord blood, dental pulp, lung, and liver. Indeed, bone marrow is among tissues severely injured by DOX, which has detrimental effect on local stem cell compartment including bone marrow-derived MSCs. Although MSCs are equipped with efficient enzymatic and non-enzymatic antioxidant mechanisms [101], accumulation of ROS can influence the growth, self-renewal, and differentiation of MSCs [102, 103]. MSCs may respond to excessive oxidative stress undergoing premature senescence with a decreased ability to secrete trophic factors [104, 105], thus having a deep impact on their anti-inflammatory and immunomodulatory properties [106]. MSCs isolated from animals subjected to DOX administration exhibited a lower proliferation rate, had a limitative capacity to respond to cardiomyogenic stimuli and when treated *in vitro* with DOX, experienced premature senescence and reduced clonogenicity [107, 108].

In the diseased heart, the participation of MSCs in the activation of the local repair machinery has been reported, but their ability to differentiate into cardiomyocytes and contribute, in a direct way, to functional recovery has not been conclusively proven [109–112]. The use of MSCs isolated from sources other than bone marrow, (e.g. adipose tissue) is relatively easy and reproducible, making this cell population a valuable tool in regenerative medicine. The transplantation of adipose tissue-derived MSCs was associated with beneficial effects on heart function after experimental myocardial infarction [113, 114], and on vascular system by promoting revascularization and tissue repair in a murine model of hindlimb ischemia [115, 116]. It is evident that a paracrine mode of action represents the main mechanism of action through which MSCs stimulate tissue repair. In fact, MSCs can produce and release a broad variety of cytokines, chemokines, and growth factors serving as supportive signaling for other cells directly involved in the repair of the injured myocardium.

4.5. Endothelial progenitor cells

The discovery that endothelial progenitor cells (EPCs) can home to the site of injury and regulate vascular repair and local angiogenesis has increased the interest in their potential

use for therapeutic application [117]. The concept that cardiovascular homeostasis requires an adequate number of functional EPCs is supported by the correlation between the number of circulating EPCs and cardiovascular events [118]. The capacity of EPCs to promote angiogenesis after a vascular insult is hampered by stress-induced cellular aging processes. DOX has been shown to affect EPC function by increasing oxidative stress and activating senescence pathways via the activation of NADPH oxidase [119]. In addition, subapoptotic dose of DOX accelerated senescent processes of EPCs by regulating p38 and JNK MAPKs and enhancing $p16^{INK4a}$-dependent signaling [120]. Therefore, ROS accumulation and induction of senescence seem to be key mechanisms implicated in the effects that DOX exerts on EPCs, thus hindering their functional capacity and leading to the failure of EPC-mediated regenerative processes.

5. Conclusion

The growing cardio-oncology discipline detects and examines cardiovascular signals that emerge from cancer therapies powering reverse-translational research. Not surprisingly, the number of molecular mechanisms and cellular phenomena is growing. This new knowledge, resulting from always more in-depth studying of anthracycline cardiotoxicity, creates an opportunity to gain new insights into myocardial biology and to identify new targets that can be valid also in cardiovascular pathologies unrelated to oncologic treatment.

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Conflict of interest

The authors declare no conflict of interest.

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Chapter 3

Doxorubicin-Induced Cardiotoxicity

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Additional information is available at the end of the chapter

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Abstract

Doxorubicin (DOX) is one of the most effective antineoplastic drugs. However, its clinical use is largely limited by potential dose-dependent cardiotoxicity. To date, the mechanisms of DOX-induced cardiotoxicity remains incompletely understood. More importantly, no efficient therapeutic strategy is available to counteract DOX-induced cardiomyopathy, underscoring the importance of the prevention of this disease. In this chapter, we first describe the pathophysiology of DOX-induced cardiotoxicity. We then update the findings of molecular biology of DOX-induced cardiomyopathy including molecular mechanisms, established and putative biomarkers for early diagnosis, and potential genetic factors for prediction of susceptibility. Finally, we introduce a number of pharmaceutical measures and practical lifestyle modifications for the prevention of this disease.

Keywords: doxorubicin, cardiotoxicity, cardiac function, oxidative stress, iron accumulation, topoisomerase, autophagy, mitochondria, inflammation, calcium, cell death, microRNA, polymorphisms, antioxidant, exercise, fasting

1. Introduction

Doxorubicin (DOX), an anthracycline antibiotic produced by the fungus Streptomyces peucetius, has been proved to be one of the most effective drugs for the treatment of solid tumor and haemotological malignancies. However, the clinical use of DOX is limited by potential dose-dependent cardiotoxicity. Incidences of progressive congestive heart failure were approximately 5, 16, 26 and 48% in patients who had received a cumulative dose of 400, 500, 550 and 700 mg/m^2 of DOX, respectively [1]. DOX-induced cardiotoxicity can be acute or chronic. Acute DOX cardiotoxicity occurs within several days after administration of the drug, while chronic DOX cardiotoxicity takes place months or even years after use of DOX [2]. However,

the biological mechanisms underlying DOX cardiotoxicity is not fully understood, although multiple factors have been suggested. As a consequence, no efficacious therapeutic strategies are available to cure DOX cardiotoxicity. Therefore, the prevention of DOX cardiotoxicity is crucial for cancer patients. Currently, several pharmaceutical strategies have been used or tested clinically to prevent DOX cardiotoxicity. In addition, a number of nonpharmacological strategies have shown promising results in preclinical studies. To accomplish more successful prevention or intervention of DOX cardiotoxicity, efforts should be exerted on identification of the susceptible population on the basis of genetic variants or early diagnosis of this disease taking advantage of biomarkers. In this chapter, we first describe morphological and functional characteristics of the heart in DOX cardiotoxicity. We then update the findings regarding molecular biology of DOX cardiotoxicity. Finally, we introduce several promising pharmacological strategies and lifestyle modifications for the prevention of DOX cardiotoxicity.

2. Morphological and functional characterization

The earliest alteration of the heart in DOX cardiotoxicity is calpain-dependent degradation of a giant cardiac structural protein titin, which may predispose the heart to diastolic dysfunction [3]. Histological changes include cardiomyocyte vacuolar degeneration and myofibrillar disarray [4]. In addition, fibrosis is markedly increased in both interstitial area of myocardium and perivascular area in animal models of chronic DOX-induced cardiotoxicity [5]. At the ultrastructural level, DOX-induced cardiac damage is characterized by dilatation of sarcoplasmic reticulum, loss of the Z-band, myofibrillar dropout, marked accumulation of cytoplasmic vacuoles, damaged mitochondria, and increased numbers of autophagic vacuoles [6, 7]. These changes result in cardiomyocyte dysfunction and cell death via necrosis or apoptosis. Cell death and fibrosis lead to compromised cardiac function in DOX-induced cardiomyopathy. DOX cardiotoxicity can be diagnosed if the patients receiving DOX treatment show signs and symptoms of congestive heart failure. However, DOX cardiotoxicity is usually diagnosed on the basis of left ventricular cardiac function. Three types of criteria are widely used to diagnose DOX cardiotoxicity: (i) the left ventricular ejection fraction (LVEF) is reduced by 20% to a value >50%, (ii) the LVEF is reduced by 10% to a value <50%, and (iii) the LVEF is reduced by >10 points to a value <50% [8].

3. Cellular and molecular mechanisms

The cause of DOX cardiotoxicity is multifactorial, and the precise mechanisms remain to be elucidated. Here, we describe the major mechanisms that have been suggested to contribute to DOX cardiotoxicity. It should be pointed out that the mechanisms are not mutually exclusive. As a matter of fact, most of the factors are interconnected with each other.

3.1. Oxidative stress

Oxidative stress, caused by enhanced intracellular levels of reactive oxygen species (ROS), has long been believed to be the major mediator of DOX cardiotoxicity. The major types of

ROS include superoxide radical (O^{2-}), hydrogen peroxide (H_2O_2), and hydroxyl free radical (HO) [9]. ROS is mainly generated through redox cycling in mitochondria [9]. However, ROS is also produced outside mitochondria by activation of pro-oxidant enzymes such as NADPH oxidase and xanthine oxidases [10]. Low level of ROS functions as signaling molecules and cell defense system. The cells have efficient antioxidant defense system to eliminate overproduced ROS and maintain ROS to physiological levels [11]. However, if the balance between ROS production and antioxidant system is disrupted in favor of ROS production, then oxidative stress occurs, which triggers a number of deleterious events including DNA damage, mitochondrial dysfunction, cell death, disrupted cellular calcium homeostasis, attenuated protein synthesis, defect in protein quality control, and mitochondrial quality control [12]. After DOX treatment, DOX is preferentially accumulated in mitochondria. As a potent electron acceptor, DOX promotes ROS generation and damages the activities of antioxidant enzymes, shifting the balance between pro-oxidant and antioxidant to the former, leading to elevated ROS levels. Excessive ROS is capable of damaging mitochondria, which in turn, produces more ROS, forming a vicious cycle called ROS-induced ROS release [13]. Given that the cardiomyoyctes are exceptionally rich in mitochondria, DOX is especially harmful to the heart. At the molecular level, the harmful effects of DOX-induced ROS are exerted primarily by its direct damage to mitochondrial genome, RNA, proteins and lipids [12]. In addition, enhanced ROS also participates in cellular signaling involved in detrimental events such as DNA damage and cell death [14].

3.2. Iron accumulation

Following DOX administration, DOX cardiotoxicity occurs through iron accumulation in mitochondria. Cardiac specific over-expression of ABCB8, a mitochondrial inner membrane protein involved in iron export, reduced iron accumulation in mitochondria and mitigated DOX cardiotoxicity [15]. Dexrazoxane, a drug approved by FDA to prevent DOX cardiotoxicity, decreased iron accumulation and ameliorate DOX-induced cardiac injuries in mice. In addition, patients with DOX cardiotoxicity showed higher levels of mitochondrial iron compared with patients with other types of cardiomyopathy or patients with normal cardiac function [15]. These studies provide convincing evidences demonstrating that iron accumulation is one of the major mechanisms involved in DOX cardiotoxicity. However, the underlying mechanisms that iron overload causes DOX cardiotoxicity remain to be clarified. Although several lines of evidences point to enhanced ROS generation by iron accumulation, a number of antioxidants fail to protect DOX cardiotoxicity in clinical settings, suggesting that other unidentified mechanisms are responsible for iron accumulation-mediated cardiac damage in DOX cardiotoxicity [16].

3.3. Topoisomerase IIβ

Type II topoisomerases (Top II) is an enzyme that generates DNA double-strand breaks, which is crucial to control the conformational changes of DNA and the entire chromosome. Mammalian cells consist of two types of Top II isoenzymes, Top IIα and Top IIβ. Top IIα is only expressed in proliferating cells, while Top-IIβ is ubiquitously expressed including postmitotic cells such as adult cardiomyocytes [17]. The antitumor activity of DOX is achieved through the formation of Top II-DOX-DNA ternary complex (also called the cleavage complex), which

increases Top II-DNA complexes and consequent DNA double-strand breaks [17]. In cardiomyocyte, Top IIβ is targeted by DOX, and the increased Top IIβ DNA cleavage complex induces DNA damage, which in turn, leads to cell death. Cardiomyocyte-specific depletion of Top IIβ conferred protection against DOX-induced DNA double-strand breaks, transcriptome changes, and heart failure [18, 19]. These data suggest that Top IIβ in cardiomyocytes plays a major role in mediating DOX-induced cardiotoxicity.

3.4. Macroautophagy dysregulation

Macroautophagy (hereafter referred to as autophagy) is a conserved pathway delivering cytoplasmic contents to lysosome for degradation and recycling [20]. Basal level of autophagy in the heart plays an essential role in the maintenance of cardiac structure and function by removing damaged protein and organelles such as mitochondria [21]. Autophagy can be either activated or suppressed in pathological conditions [22]. The significance of autophagy activation can be either beneficial or detrimental depending upon pathological settings [22]. Recent studies have shown that autophagy is dysregulated after DOX treatment in animals. However, it is controversial whether autophagy is activated or suppressed. There are studies showing that DOX treatment activates autophagy in the heart or cardiomyocytes [23–26], while others have shown conflicting results [7, 27–30]. Moreover, the significance of autophagy in DOX cardiotoxicity is still on debate. Some data are in favor of beneficial effects of autophagy in DOX cardiotoxicity [23–26], while others argue against it [27–30]. The discrepancies may be caused by the difference in animal species, cell types, methods monitoring autophagy, means of drug administration, and dosage and duration of the drug used in these studies. More recently, we and others have shown that DOX treatment stimulated autophagy initiation, while suppressed multiple subsequent steps including autophagosome formation, autophagosome maturation and lysosomal degradation [7, 27, 29, 30]. As a consequence, the autophagic flux was attenuated in DOX-induced cardiotoxicity. Inhibition of autophagic flux using UVRAG-deficient mice exacerbated DOX-induced cardiotoxicity [30]. Conversely, enhancement of autophagic flux mitigated DOX cardiotoxicity [27, 29, 30]. In addition, suppression of autophagy initiation using *beclin* $1^{+/-}$ mice ameliorated DOX cardiotoxicity [7]. The regulation of autophagy in the heart and its significance in cancer patients treated by DOX needs to be investigated. Moreover, the effects of autophagy modulation on cancer cells should be considered if autophagy is targeted for prevention of DOX cardiotoxicity.

3.5. Mitochondrial dysfunction

Mitochondria are the organelle that produces ATP, which plays an essential role in cell survival. Mitochondria are the major source of free radicals and as a consequence are vulnerable to damage caused by oxidative stress. It has been demonstrated that mitochondrial dysfunction is one of the mechanisms of DOX cardiotoxicity [12]. Under physiological conditions, mitochondrial quality is controlled by mitochondrial quality control system, which includes selective elimination of mitochondria by autophagy (also called mitophagy), mitochondrial biogenesis, and mitochondrial dynamics including mitochondrial fusion and fission [31].

Pink1-Parkin-mediated mitophagy is the most well-studied mechanism for mitophagy. Pink 1 is a serine/threonine kinase, which is normally localized in the inner membrane of mitochondria (IMM). However, in depolarized mitochondria, Pink 1 is unable to be translocated to IMM and is retained on the outer membrane of mitochondria (OMM), where Pink-1 undergoes autophosphorylation and is activated. The activated Pink-1 then recruits parkin, a cytosolic E3 ligase to the OMM. Parkin ubiquitinates the substrate proteins localized on the OMM and facilitates degradation of mitochondria by autophagy [32, 33]. DOX treatment has been shown to suppress Pink 1 and Parkin expression [34]. In addition, DOX enhances p53 expression, which promotes its interaction with Parkin and prevents Parkin translocation from cytoplasm to mitochondria [35]. Moreover, as aforementioned, DOX inhibits autophagic flux in the heart at multiple steps, which also attenuates mitochondrial degradation [7, 27, 29, 30]. Therefore, DOX treatment suppresses Pink 1-Parkin-mediated autophagy in the heart and promotes accumulation of damaged mitochondria. In addition to Pink 1-Parkin-meidated mitophagy, other mitochondria-localized proteins such as Nix, Bnip3, FUNDC1, and cardiolipin have been shown to interact with LC3 or LC3 homologs to mediate mitophagy [33]. However, the significance of Parkin-independent mitophagy mediated by these molecules remains to be elucidated in DOX cardiotoxicity.

Mitochondria are highly dynamic organelle, which continuously undergo fusion and fission to organize interconnecting networks to fulfill its function. Mitochondrial fusion and fission are essential for the maintenance of mitochondrial number and quality under stress conditions. Mitochondrial fusion allows the mixture of the contents from partially damaged mitochondria and healthy mitochondria to alleviate the stress. Mitochondrial fission separates mitochondria into two daughter mitochondria, which allows the biogenesis of new mitochondria and the removal of the damaged mitochondria via mitophagy [31]. Mitochondrial fusion is controlled by GTPase Mitofusin1 (MFN1), Mitofusin2 (MFN2), and optic atrophy factor 1 (OPA1). MFN1 and MFN2 are localized to the OMM, while OPA1 is an IMM protein. MFN1, MFN2, and OPA1 mediate the fusion of the OMM and IMM, respectively [31]. Mitochondrial fission is mainly regulated by Drp1, a large GTPase. Drp1 is recruited from cytoplasm to mitochondrial OMM during fission process. In mitochondrial OMM, Drp1 has four interacting partners, FIS1, Mff, Mid55, and Mid49 [31, 36]. Mitochondrial fusion and fission are well balanced to maintain mitochondrial number and quality under physiological conditions. In animal models of DOX cardiotoxicity, DOX treatment induces changes in the expression of mitochondrial fusion and fission proteins, which alters mitochondrial dynamics and contributes to apoptosis [37].

Mitochondrial biogenesis is the process of expansion of existing mitochondria or generation of new mitochondria. Mitochondrial biogenesis is tightly regulated to coordinate mitophagy, mitochondrial fusion and fission for the maintenance of mitochondrial mass and remodeling of dynamic interconnected mitochondrial network. DOX treatment impairs cardiac mitochondrial biogenesis as manifested by reduced mitochondrial DNA copy number and expression of regulating factors for mitochondrial biogenesis such as peroxisome proliferator-activated receptor gamma coactivator 1-alpha, peroxisome proliferator-activated receptor alpha, and estrogen-related receptor alpha, leading to suppression of mitochondrial metabolism and ATP synthesis [38, 39].

3.6. Inflammation

A growing body of evidences has shown that cardiac inflammation contributes to DOX cardiotoxicity. DOX treatment induces increased activity of NF-κB, a key component of innate immune system, leading to enhanced levels of pro-inflammatory cytokines including IL-1β, IL-6, and TNFα [40]. Toll-like receptors (TLRs) especially TLR2 has been considered as the major mediator to activate NF-κB [40]. DOX-induced oxidative stress and damage-associated molecular pattern molecules (DAMPs) such as HMGB-1 are responsible for the activation of TLR2 [41]. In addition to TLR2, TLR9 is capable of activating NF-κB and may be engaged in cardiac inflammation in DOX-induced cardiotoxicity [42]. It has been shown that mitochondrial DNA escaped from autophagy triggers cardiac inflammation through TLR9 activation during progression of pressure-overloaded heart failure [43]. Given that autophagic flux in the heart is impaired by therapeutic dose of DOX, it is likely that TLR-9 activation is involved in inflammatory response in DOX-induced cardiotoxicity. However, studies need to be designed to address this issue.

3.7. Abnormal intracellular calcium handling

Calcium is critical for cardiac systolic and diastolic function. Calcium regulates cardiac contraction through a process called cardiac excitation-contraction coupling (EC coupling). In this process, calcium enters cytoplasm through L-type calcium channel activates ryanodine (RyR) receptor localized on the sarcoplasmic reticulum (SR) membrane, resulting in calcium-induced calcium release in the SR. The released calcium form SR stimulates cardiomyocytes to contract. Subsequently, the cytoplasmic calcium is taken up by the sarcoendoplasmic reticulum calcium transport ATPase (SERCA2) localized on the SR membrane, resulting in reduced cytoplasmic calcium concentration and cardiomyocyte relaxation [44]. DOX regulates cytoplasmic calcium levels through several mechanisms. First, DOX is able to bind RYR2 directly and enhances its open probability [45]. Second, DOX is capable of interacting with calsequestrin, a calcium binding protein localized in SR lumen, and promotes calcium release [46]. Third, DOX elevates intracellular calcium levels by binding to SERCA2A and modify its activity [47]. Fourth, DOX induces SR calcium leakage in a CAMK II-dependent manner, leading to impaired calcium handling in cardiomyocytes [48]. Finally, oxidative stress induced by DOX amplifies RYR opening and calcium release [49]. Thus, DOX regulates calcium release from SR through both oxidant-dependent and independent mechanisms, and the abnormal calcium handling contributes to DOX cardiomyopathy.

3.8. Cell death

Numerous studies have shown that DOX induces apoptosis, which contributes to cardiotoxicity. DOX stimulates ROS generation and produces oxidative stress, which activates p53. In addition, DOX itself promotes p53 activity in the heart. p53-mediated signaling stimulates apoptotic cell death of cardiomyocytes [50, 51]. Moreover, multiple lines of evidences have suggested that mitochondrial calcium is overloaded and contributes to apoptotic cell death of cardiomyocytes in DOX cardiotoxicity. As aforementioned, DOX promotes calcium release

from SR. Mitochondria, which are physically close to SR calcium release sites, uptake a portion of calcium released from SR, leading to rise in mitochondrial calcium levels. Calcium overload triggers loss of mitochondrial membrane potential, swelling of mitochondria, and ultimately rupture of OMM and leakage of cytochrome C, resulting in apoptosis of cardiomyocytes [52].

Necrotic cardiomyocyte death is also increased in DOX cardiotoxicity. Oxidative stress induced by DOX is considered as the major cause for necrosis. Oxidative stress enhances calcium release from SR and raises calcium levels in mitochondria, which induces loss of mitochondrial membrane potential, mitochondrial swelling, and ultimately mitochondrial outer membrane rupture, leading to ATP depletion [53]. In addition, oxidative stress induces mitochondrial DNA damage and mitochondrial lipid peroxidation, leading to disruption of integrity of mitochondrial structure, mitochondrial dysfunction, and ATP depletion [54]. Recently, Bnip3 has been shown to disrupt interaction of COXI and UCP3, leading to defective mitochondrial respiratory chain and cardiomyocyte necrosis in DOX cardiotoxicity [55].

4. Biomarkers and genetic factors

Currently, no effective therapy is available to cure DOX-induced cardiotoxicity. Thus, prevention become more important and should be primarily directed. Early detection is crucial for the prevention of irreversible cardiac damage. Traditional technology such as echocardiography, electrocardiogram, and angiography are not efficient for early detection of cardiac damage since cardiac dysfunction already occurs when diagnosis is made by means of aforementioned technology. Biochemical biomarkers are sensitive and ideal for early detection of cardiac damage. Two types of biomarkers, i.e., troponins and natriuretic peptides, have been established and are currently used in clinic for early diagnosis of DOX cardiotoxicity. In addition, other promising putative biomarkers have been tested.

4.1. Cardiac troponins and B-type natriuretic peptide

Cardiac troponins are a complex consisting of three regulatory proteins, i.e., troponin T (cTnT), troponin C (cTnC), and troponin I (cTnI) in cardiac muscle. cTnT and cTnI are well-established sensitive and specific biomarkers to detect myocardial damage caused by differential insults [56]. Both cTnI and cTnT have also been utilized in clinic to detect and predict cardiac damage caused by DOX [57, 58].

B-type natriuretic peptide (BNP) is a peptide prohormone, which is primarily produced in ventricles and brain. BNP is synthesized as pre-pro-BNP, which is cleaved to generate pro-BNP. Pro-BNP is further cleaved into a C-terminal biologically active form of BNP and N-terminal inactive form of NT-pro-NPs. Both NT-pro-NPs and BNP are secreted into serum and serve as sensitive biomarkers predictive of congestive heart failure [59–61]. Currently, NT-pro-NPs and BNP are used in clinic as indicators of early cardiac damage caused by DOX [62, 63].

4.2. MicroRNAs

MicroRNAs can become ideal clinical biomarkers due to their characteristics such as high stability, tissue specificity, and presence in body fluids [64]. Emerging evidences have indicated that alteration of certain microRNAs is associated with DOX cardiotoxicity and may be served as biomarkers. An in vitro study using human pluripotent stem cell-derived cardiomyocytes showed that a number of microRNAs, including miR-34a, miR-34b, miR-187, miR-199a, miR-199b, miR-146a, miR-15b, miR-130a, miR-214, and miR-424, were differentially expressed during and after DOX treatment [65]. However, the expression pattern of these microRNAs in animal models and patients receiving DOX treatment remains to be investigated. A study using a mouse model of DOX cardiotoxicity explored whether microRNAs including miR-208a, miR-133b, miR-146a, miR423-5p and miR-1 are suitable to predict cardiac damage in patients receiving DOX treatment. The results showed that miR-208a and miR-208b were not useful biomarkers for DOX cardiotoxicity since they were undetectable in the serum. MiR-133b, miR-146a, and miR423-5p were not appropriate biomarkers either since although detectable, no significant alterations were observed in cardiotoxic-patients compared with noncardiotoxic-patients. miR-1 was upregulated in patients suffering from cardiotoxicity compared with noncardiotoxic patients. Moreover, miR-1 expression levels were associated with changes of left ventricular ejection fraction. Therefore, miR-1 is a promising circulating biomarker for early detection of cardiac injury caused by DOX [66]. However, further studies should be developed to validate the putative diagnostic marker.

4.3. Genetic risk factors

The susceptibility to DOX cardiotoxicity is apparently patient dependent, suggestive of a role of genetic factors. To date, a number of gene polymorphisms associated with DOX cardiotoxicity have been identified. A German non-Hodgkin lymphoma study including 1697 enrolled patients has suggested that polymorphisms of the NAD(P)H oxidase were associated with DOX cardiotoxicity. Specifically, the 212A→G variant of NAD(P)H oxidase subunit NCF4 was associated with chronic DOX cardiotoxicity. The His72Tyr polymorphism in the p22phox subunit and the variant 7508T→A of the RAC2 subunit of NAD(P)H oxidase were associated with acute DOX cardiotoxicity [67]. Consistent with these findings, mice deficient for NAD(P)H oxidase activity were resistant to chronic doxorubicin treatment [67]. In the same study, Gly671Val variant of the doxorubicin efflux transporter multidrug resistance protein 1 (MRP1) and the Val1188Glu-Cys1515Tyr haplotype of MRP2 have been shown to be associated with acute DOX cardiotoxicity [67]. Polymorphisms of other genes that have been reported to be potentially associated with cardiotoxicity caused by DOX or DOX-based treatment include CBR3, CAT, ABCB1, ABCC1, ABBCC2, RAC2, GSTP1, CYBA, ABCC5, CASP3, MSH2, SLCO1A2, SLC28A3, FMO2, SPG7, SLC10A2, UGT1A6, ABCB4, SULT2B1, HFE, POR, HAS3, HNMT, SLC22A7, SLC22A17, RARG, and NOS3 [68]. Most of the candidate genes are related to cellular transport of DOX, oxidative stress, DOX metabolism, and DNA repair and replication. In a recent study involving a relatively small number of patients treated with DOX for breast cancer, 18 SNPs in nine genes in the HLA region (NFKBIL1, TNF-α, ATP6V1G2-DDX39B, MSH5, MICA, LTA, BAT1, and NOTCH4) and in the psoriasis

susceptibility region of HLA-C were identified to be potentially associated with DOX cardiotoxicity, implicating an important role of dysregulation of genes involved in inflammatory disease and autoimmune disorders in DOX cardiotoxicity [69]. Polymorphisms of RAAS genes, which are useful for the prediction of congestive heart failure, were not significantly associated with DOX-induced cardiotoxicity [67]. Additional studies are required to identify and functionally validate genetic variants in DOX cardiotoxicity.

5. Preventive strategy

5.1. Doxorubicin dosage and administration

Given that DOX-induced cardiotoxicity is cumulative dose-dependent, the most straightforward way to prevent DOX cardiotoxicity is to reduce the dosage utilized for patients. However, lower dosage is associated with less therapeutic efficacy [70]. Thus, alternative approaches of drug administration such as continuous infusion and liposome DOX versus bolus injection are used to prevent cardiac toxicity. Continuous infusion of DOX causes significantly less injury to the heart compared to bolus doses without compromising cancer treatment efficacy. The mechanisms are due to the changes in the distribution of DOX with reducing drug concentration in the heart and no impact on drug doses in tumor tissues [71–73]. It should be pointed out that continuous infusion does not confer cardiac protection in children with acute lymphoblastic leukemia [74]. Administration of DOX by liposome encapsulation is another effective strategy to reduce cardiotoxicity. Liposomal DOX formulation is not capable of crossing the tight gap junction of endothelial cells of blood vessels in the heart. However, in tumor tissues, the vasculature is irregular and leaky, which allows the diffusion of liposomal DOX formulation [75]. In addition, the diffused DOX accumulates in the tumor tissue due to poor lymph drainage. Both lead to selective accumulation of DOX in tumor tissues. This phenomenon is known as "enhanced permeability and retention effect," which characterizes solid tumors and is used to target tumor cells [76]. Moreover, the liposomal DOX formulations diffused into tumor tissues are prone to destabilization due to more acidic extracellular pH, release of necrotic tumor cell lipases, and inflammatory cell oxidizing agents in tumor microenvironment [76]. A number of preclinical and clinical studies have demonstrated that liposomal DOX formulation delivers relatively larger amount of DOX to tumor tissues and much less doses to the heart tissues compared to conventional DOX. Thus, the liposomal DOX formulations are more active and safer. Currently, two types of liposomal DOX formulations, i.e., pegylated (Caelyx® in Europe and Doxil® in the USA) or nonpegylated (Myocet®), have been approved as a first-line treatment for defined group of cancer patients [77]. In recent years, nanoparticle DOX delivery systems have attracted much attention due to potential increased bioavailability in tumor tissues and minimum cardiac toxicity, which hold promise as an efficient approach for the prevention of DOX cardiotoxicity [78].

DOX treatment combining with cardioprotective agents is an alternative strategy to prevent cardiotoxicity. Dexrazoxane (Zinecard, ICRF-187, ADR-529, NSC-169780), a cyclic derivative of edetic acid, is a cardioprotective agent approved by FDA to prevent DOX cardiotoxicity in

the clinic [79]. The molecular mechanisms that Dexrazoxane confers cardioprotection have previously been attributed to its iron chelating capability. However, other iron chelators fail to exert preventive effects for DOX cardiotoxicity, suggesting that iron chelation is not the major molecular basis for dexrazoxane cardioprotection. It turns out that dexrazoxane interferes with Top IIβ either through promoting Top IIβ proteasomal degradation or preventing the formation of Top IIβ-DNA cleavage complex in cardiomyocytes [79]. It should be noted that coadministration of dexrazoxane may trigger secondary malignancies in cancer patients [80]. However, this issue is still controversial and requires further investigation.

5.2. Antioxidant reagents

Considering oxidative stress has been believed to be the major mediator of DOX-induced cardiotoxicity, it is reasonable to expect that coadministration of antioxidants is capable of preventing or mitigating DOX cardiotoxicity. The antioxidants reduce intracellular ROS levels through reducing ROS generation, scavenging ROS themselves, chelating irons to inhibit HO. formation or eliminating other active molecules generated in response to ROS reaction such as lipid peroxide [81]. Although antioxidants are effective in the treatment of acute DOX cardiotoxicity in animal models, Clinically relevant animal experiments and clinical trials have suggested that among a variety of antioxidant reagents, only dexrazoxane has shown definitive effect on DOX cardiotoxicity [79]. As mentioned above, dexrazoxane ameliorates DOX cardiotoxicity likely through mechanisms independent of ROS elimination [79]. Thus, it still remains unclear whether antioxidants should be given to cancer patients during or after DOX treatment to prevent cardiotoxicity. In addition, ROS generation could be the mechanism that DOX is toxic to cancer cells, antioxidant may reduce response rate for DOX in patients, although DOX may cause cytotoxicity in cancer cells through both ROS-dependent and independent mechanisms. Further study should be conducted to address these issues.

5.3. Neurohormone blockers

Neurohormone blockers such as angiotensin II-converting enzyme inhibitors and angiotensin receptor blockers have been widely utilized in clinics to treat heart failure including DOX-induced heart failure. Angiotensin receptor blockers have been shown to prevent decline of cardiac function induced by DOX in cancer patients. The preventive effect may be related to decreased generation of oxidative stress and reduced apoptosis of cardiomyocytes [82, 83]. Thus, neurohormone blockers may be used in combination with DOX to prevent cardiac toxicity.

5.4. Exercise

In addition to pharmaceutical measure, lifestyle modifications are promising alternative strategies to counteract DOX-induced cardiomyopathy since it is practical to be introduced to patients. Several types of exercise such as chronic resistance exercise [84], chronic swimming [85], voluntary exercise [86, 87], and treadmill running [88–91] have been shown to exert beneficial effect on mitigation of cardiac structural damage and preservation of cardiac

performance in animal models of DOX cardiotoxicity. Moreover, acute exercise prior to DOX treatment protects cardiac function of breast cancer patients [92]. The protective effects of exercise on DOX-induced cardiac injury may be attributed to increased antioxidant ability, increased expression of heat shock proteins and antiapoptotic proteins, improved mitochondrial quality control, maintenance of calcium handling, and altered delivery of DOX to myocardium [90, 91, 93]. Importantly, exercise training has no effect on antitumor efficacy of DOX [94]. However, these preclinical and clinical findings need to be verified by studies involving a large cohort of patients.

5.5. Calorie restriction and fasting

Calorie restriction is beneficial for several types of cardiovascular diseases including DOX cardiotoxicity [95, 96]. However, calorie restriction is hard to sustain in the long term. Although calorie restriction mimetics are more practical in terms of sustainability, they are less accessible and cost ineffective. Fasting has been shown to exert beneficial effects on certain forms of cardiovascular diseases including age-related cardiac hypertrophy, myocardial ischemic injury, and coronary heart disease risk factors through diverse mechanisms including remodeling of mitochondrial networks, improvement of energy metabolism, reduction in signaling pathways related to survival such as insulin and insulin-like growth factor-1 signaling, decrease in mitochondrial oxidative stress, and enhancement of autophagic flux [97, 98]. Recent studies suggest that fasting also conferred cardioprotection against DOX cardiotoxicity. In animal models, short-term fasting ameliorates cardiac damage and cardiac dysfunction caused by DOX [98]. Alternate-day fasting, a type of intermittent fasting, is capable of mitigating DOX cardiotoxicity in mouse models of both acute and chronic DOX cardiotoxicity [30]. More importantly, intermittent fasting and multiple fasting cycles have recently been shown to suppress tumor growth and sensitize various tumors to chemotherapy [99, 100]. Therefore, intermittent fasting could be considered as a potential preventive or therapeutic strategy for cardiotoxicity induced by DOX. However, given that long-term fasting is harmful to health especially for cancer patients due to malnutrition problem, the procedure of intermittent fasting should be optimized under clinical supervision to improve its efficacy while minimizing side effects.

6. Conclusions

DOX is one of the most effective chemotherapeutic agents. However, potential acute or chronic irreversible cumulative cardiotoxicity limits its clinical application. It is encouraging that accumulating evidences from basic research, preclinical experiments and clinical trials provide insight into the pathophysiology and molecular mechanisms of this disease, which potentially leads to identification of novel biomarkers for early detection and establishment of preventive strategies. Moreover, emerging evidences have associated DOX cardiotoxicity with genetic risk factors. Findings in this direction will be helpful to predict tumor sensitivity to DOX treatment and susceptibility to DOX-induced cardiotoxicity of the population. As

a consequence, precise strategies may be developed and applied to individuals to achieve maximal efficacy for cancer treatment and meanwhile minimal side effects on the basis of patient-specific genetic variants.

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Conflict of interest

No potential conflict of interests were declared.

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Section 2

Basic Research: Non-Anticancer Agents Associated Cardiotoxicities

Chapter 4

Thiazolidinediones Cause Cardiotoxicity via PPARγ-Independent Mechanism

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Additional information is available at the end of the chapter

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Abstract

Thiazolidinediones (TZDs), peroxisome proliferator-activated receptor gamma (PPARγ) agonists, are highly effective antidiabetic drugs that are widely used to treat type 2 diabetes mellitus (T2DM) due to their unique beneficial actions, such as a renoprotective effect, amelioration of glucose homeostasis, and blood pressure lowering, that other antidiabetic drugs do not have. Those beneficial actions, however, are shadowed by the increased risks of cardiovascular adverse events, including mitochondrial dysfunction, oxidative stress and myocardial energy deficiency, fluid retention, congestive heart failure, and myocardial infarction. Except PPARγ, TZDs also have affinity to numerous non-PPARγ targets in mitochondria, cytosol, and cytoplasm, including MitoNEET, mitochondrial pyruvate carrier, dehydrogenases involved in tricarboxylic acid cycle and electron transport, cytoplasmic ion channels, Na-K-pump, and other unknown enzymes. By binding to these targets, TZDs produce off-target effects and potentially increase cardiotoxicity. In this chapter, we review recent studies, both experimental and clinical, on the myocardial adverse effects associated with TZDs and their underlying mechanisms. We focus our review in large part on the relationship between these myocardial adverse effects and PPARγ.

Keywords: thiazolidinedione, myocardial energy metabolism, mitochondria, oxidative stress, peroxisome proliferator-activated receptors, heart failure, myocardial infarction

1. Introduction

Thiazolidinediones (TZDs) including ciglitazone, pioglitazone, rosiglitazone, and troglitazone, also known as glitazones after the prototypical drug ciglitazone, are a class of

heterocyclic compounds consisting of a five-membered C3NS ring. Among them, pioglitazone and rosiglitazone were approved for clinical use in the United States and Canada. Through activation of peroxisome proliferator-activated receptor gamma (PPARγ), these compounds improve insulin sensitivity, reduce hyperglycemia, and afford unique beneficial actions, such as a renoprotective effect and blood pressure lowering that other antidiabetic drugs do not have [1]. Therefore, they have been widely used to treat type 2 diabetes mellitus (T2DM) as monotherapy or in combination with other types of oral antidiabetic agents (sulfonylureas, metformin, and acarbose). Their original approvals were based on the ability to reduce insulin resistance, increase peripheral glucose utilization, and decrease hepatic glycogen output, accordingly, lower blood glucose concentration [2]. TZDs provide robust improvement in glycemic control that is comparable to other established agents, such as metformin and the sulfonylureas [3, 4]. More importantly, since the progressive failure and loss of β-cells are ultimately responsible for the onset and progression of T2DM, the potential of TZDs to preserve β-cells is an extremely desirable function in glucose-lowing medicine [5–7]. According to ADOPT (A diabetes Outcome Prevention Study), the rate of monotherapy failure with TZDs is lower than other antidiabetic agents such as metformin and glyburide [8].

The ultimate value of TZDs and any other glucose-lowing drugs should rely on not only the improvement of acute hyperglycemic crises and their serious consequences, but also the reduction of long-term complications associated with diabetes. Theoretically, reducing hyperglycemia over the long term should decrease the possibility of the complications, but this is not the case for rosiglitazone. Instead, its beneficial actions are shadowed by the increased risks of cardiovascular adverse events [9].

The coexistence of heart failure (HF) and T2DM is common and has a strong impact on clinical management and prognosis. The action of any antidiabetic therapy on cardiovascular system is particularly important because more than 70% of deaths in diabetic patients are from cardiovascular causes [10], and clinical courses of cardiovascular events and T2DM frequently progress in parallel [11, 12]. Glucose-lowering drugs, including the TZDs, have complex organ-specific effects on diverse biological processes that may determine the effects on cardiovascular events end point. Unfortunately, the potential for unexpected cardiovascular side effects when rosiglitazone is administered to patients was not fully assessed before the approval from U.S. Food and Drug Administration (FDA) in 1999 and from the European Medicines Evaluation Agency (EMEC) in 2000. According to over 40 clinical trials conducted from 1999 to 2007, rosiglitazone has been reported to increase risks of heart failure [13, 14] and myocardial infarction in T2DM patients [15–17]. Additionally, rosiglitazone was associated with a significant increase in the risk of death from cardiovascular causes that had borderline significance [13, 16, 18, 19]. Rosiglitazone may also worsen the clinical course in patients with pre-existing left ventricular dysfunction [20]. Approximately 10 years after the introduction of rosiglitazone, EMEC required two post-marketing studies on long-term adverse effects and recommended that rosiglitazone be suspended from the European market because the benefits no longer outweighed the risk. Similarly, pioglitazone therapy was also associated with an increased risk of major adverse cardiovascular events in patients with pre-diabetes or insulin resistance and diabetes [21]. In this chapter, we review recent studies, both experimental and clinical, on the myocardial adverse effects associated with TZDs, rosiglitazone in

particular, and discuss their underlying mechanisms. We focus our discussion in large part on the relationship between these myocardial adverse effects and PPARγ.

2. On-target effects of TZDs

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors of nuclear hormone receptor superfamily comprising three subtypes such as PPARα, PPARβ/δ, and PPARγ [22]. PPARα is predominantly expressed in metabolically active tissues such as the adipose tissue, liver, heart, kidney and skeletal muscle [23], and mainly influences fatty acid metabolism and its activation lowers lipid levels [24]. PPARβ/δ is ubiquitously expressed with the highest levels in the intestine, colon, skin, adipose tissue, skeletal muscle and brain and kidney [25] and is involved in fatty acid oxidation in muscle [26]. PPARγ is mostly expressed in adipose tissue, but also found in the skeletal muscle, liver, kidney, colon,

Cells	Target genes	Gene expression	On-target effects	Reference
Adipocyte	Acetyl-CoA synthetase	↑	↑ triglyceride	[31]
Adipocyte	Adipocyte-specific fatty acid binding protein	↑	↑ lipid oxidation	[32]
Adipocyte	Cbl-associated protein	↑	↑ insulin sensitivity	[33]
Adipocyte	Fatty acid translocase	↑	↑ fatty acid uptake	[34]
Hepatocyte, β-cell	Glucokinase	↑	↑ glucose homeostasis	[35, 36]
Hepatocyte, β-cell	Glucose transporter 2	↑	↑ glucose sensing	[35]
Adipocyte	Glucose transporter 4	↑	↑ glucose uptake	[37]
Adipocyte	Glycerol kinase	↑	↓ free fatty acid	[38, 39]
Adipocyte	Insulin receptor substrate 2	↑	↑ insulin sensitivity	[40]
Adipocyte, hepatocyte	Interleukin-6	↓	↑ insulin sensitivity	[41, 42]
Adipocyte	Leptin	↓	↑ insulin sensitivity	[43, 44]
Adipocyte, muscle cell	Lipoprotein lipase	↑	↓ triglyceride	[45, 46]
Adipocyte	Perilipin	↑	↓ free fatty acid	[47, 48]
Adipocyte, hepatocyte	Phosphoenolpyruvate carboxykinase	↑	↑ triglyceride ↓ lipid oxidation	[30, 32, 49]
Adipocyte, hepatocyte	Tumor necrosis factor-α	↓	↑ insulin sensitivity	[41]

Table 1. Selected PPARγ on-target effects of TZDs.

intestine, pancreas, brain, immune cells, and retina and throughout the cardiovascular system at relatively low levels [24, 25, 27]. Activation of PPARγ causes lipogenesis, adipocyte differentiation, and insulin sensitization and enhances glucose metabolism and also decreases plasma free fatty acid level [26]. Functionally, this receptor controls the expression of networks of genes involved in adipogenesis, lipid and glucose metabolism, inflammation and maintenance of metabolic homeostasis. TZDs act by activating PPARγ. When activated, PPARγ and retinoid X receptor (RXR) form heterodimeric complex PPARγ /RXR, which then binds to a specific DNA sequence element termed peroxisome proliferator response element (PPRE), increasing transcription of various involved genes and decreasing transcription of others [28, 29]. The major effects of expression and repression of the aforementioned genes are to increase the storage of fatty acids in adipocytes, thereby decreasing the amount of fatty acids present in circulation. Accordingly, cells become more dependent on the oxidation of carbohydrates, more specifically glucose, in order to yield energy for other cellular processes. Therefore, TZDs improve insulin sensitivity and reduce hyperglycemia [30]. PPARγ on-target effects of TZDs are summarized in **Table 1**.

Besides, TZDs selectively augment or partially mimic certain actions of insulin, causing a slowly generated hypoglycemic effect without increasing pancreatic insulin secretion in non-insulin-dependent diabetic patients via the activation of PPARγ, which increases transcription of certain insulin-sensitive genes [2]. Thus, the action of TZDs is often accompanied by a reduction in circulating concentrations of insulin, triglycerides and non-esterified fatty acids while the β-cell function is largely restored. The PPARγ on-target effects of TZDs, however, are still not completely clear.

3. Off-target effects of TZDs

Drugs exert desired and undesired effects based on their binding interactions with protein target(s) and off-target(s), providing evidence for its efficacy and toxicity. Many different and seemingly unrelated side effects have emerged during the development of TZDs, such as fatal hepatotoxicity, rhabdomyolysis, nephrotoxicity, multisystem organ failure, etc. In isolated hearts [50] or in vivo [25], rosiglitazone suppressed Jun NH_2-terminal kinase and activated the adenosine monophosphate-activated protein kinase and protein kinase B (AKT) pathways, and these effects could not be fully blocked by a PPARγ antagonist [51]. Similarly, studies in the pig demonstrated rapid effects of troglitazone in the recovery of left ventricular function after ischemia/reperfusion injury [52, 53]. The effect of troglitazone in this model was found to be imparted not by the TZD moiety but by its tocopherol moiety, which does not activate PPARγ [53]. Together these data suggest that in addition to PPARγ-dependent (on-target) effects, TZDs also exert PPARγ-independent (off-target) effects.

Using [^{3}H]pioglitazone, a structurally related iodinated photoaffinity probe, mass spectrometry analysis and amino-terminal sequencing, a 17-kDa mitochondrial protein mitoNEET has been identified as a saturable and specific binding site for [^{3}H]pioglitazone [54]. MitoNEET is broadly expressed in insulin-sensitive tissues including the liver, muscle, adipose, and heart [55]. MitoNEET is an integral iron-sulfur-cluster transfer protein in the outer mitochondrial

membrane that has been shown to inhibit mitochondrial iron transport, which may in turn decrease mitochondrial respiratory activity [56, 57], oxidative capacity [55] and redox-sensitive signaling [58]. Overexpressing mitoNEET in adipocytes decreased the levels of reactive oxygen species [57]. In contrast, knocking down mitoNEET in adipocytes increased reactive oxygen species-induced protein damage [57].

Similarly, the mitochondrial pyruvate carrier 2 (Mpc-2) has also been identified as a direct mitochondrial target of the TZDs (mTOT) using photoaffinity and mass spectrometry-based proteomics approaches [59]. Two mTOT-binding TZDs with little effect on PPARγ (MSDC-0160 and MSDC-0602) were shown to enhance brown adipose tissue formation and improve insulin sensitivity in mice, whereas the deletion of the Mpc-2/mTOT gene resulted in a loss of brown adipose tissue formation [59]. A phase IIb study in patients with diabetes suggested that MSDC-0160 may have similar glucose-lowering efficacy to pioglitazone, with preliminary hints of fewer side effects [60]. MSDC-0160 was associated with a lower level of fluid retention [60]. These data suggest that specifically targeting Mpc-2/mTOT may have potential as a therapy for diabetes, and that both on-target and off-target effects may contribute to efficacy of the drugs, but off-target effects potentially increase cardiotoxicity.

Pioglitazone and rosiglitazone possess a common functional core, glitazone, which is considered a privileged scaffold upon which to build a drug selective for a given target-in this case, PPARγ. A retrospective analysis of pioglitazone and rosiglitazone has identified numerous non-PPARγ proteins as high affinity binders of TZDs in the rat heart, including mitochondrial and cytoplasmic dehydrogenases, ion channels, modulators and enzymes involved in glucose homeostasis, mitochondrial energy production and synaptic transduction [61].

Defining the off-target effects of TZDs and determining whether their cardiovascular adverse effects are mediated through PPARγ-dependent or -independent mechanisms will be critical in developing new therapeutic agents. From this point of view, we discuss recent studies, both experimental and clinical, on the myocardial adverse effects associated with TZDs, particularly rosiglitazone and their underlying mechanisms, focusing in large part on PPARγ-independent (off-target) mechanism in the following context.

4. Rosiglitazone causes myocardial energy deficiency and mitochondrial dysfunction via PPARγ-independent mechanism

Using ^{31}P-nuclear magnetic resonance (NMR) spectroscopy, we measured intracellular phosphocreatine (PCr), adenosine triphosphate (ATP), and calculated free energy of ATP hydrolysis (ΔG_{ATP}) in isolated beating hearts perfused in Langendorff mode with regular Krebs-Henseleit buffer containing 10 mM glucose and 0.5 mM pyruvate. At baseline, all hearts from cardiomyocyte-specific PPARγ deficient (PPARγ$^{-/-}$) mice and their littermate control (PPARγ$^{+/+}$) mice showed similar PCr and ATP resonance areas, and PCr/ATP ratio, indicating the loss of regulatory action of cardiomyocyte PPARγ on myocardial energy metabolism can be compensated in vivo. At the human therapeutic concentrations of 1 and 3 μM, rosiglitazone showed no marked effects on the resonance areas and concentrations of intracellular PCr

([PCr]) and ATP ([ATP]). At the supratherapeutic concentrations of 10 and 30 μM, however, rosiglitazone decreased myocardial [PCr], [ATP], and ΔG_{ATP} in both PPAR$\gamma^{-/-}$ and PPAR$\gamma^{+/+}$ mice in parallel compared with their vehicle controls [62]. To confirm the results from ^{31}P-NMR spectroscopy, we freeze-clamped hearts from those mice at the end of each experiment and then measured total ATP, ADP, AMP content using HPLC and calculated energy charge. Consistent with the abovementioned results, total ATP content, ATP to ADP ratio and energy charge decreased following acute treatment with rosiglitazone at 10 and 30 μM in hearts from both PPAR$\gamma^{-/-}$ and PPAR$\gamma^{+/+}$ mice compared with vehicle control [62].

Since mitochondrial oxidation of fatty acid and glucose is a major source of ATP in cardiomyocytes, we measured glucose and palmitate oxidation rates in fresh tissue homogenates using [1-^{14}C]-glucose and [1-^{14}C]-palmitic acid, respectively. At the therapeutic concentrations of 1 and 3 μM, incubation of rosiglitazone with myocardial homogenates for 60 min did not change glucose and palmate oxidation rates. At the supratherapeutic concentrations of 10 and 30 μM, however, it decreased oxidation rates of glucose and palmitate in myocardial homogenate from both PPAR$\gamma^{-/-}$ and PPAR$\gamma^{+/+}$ mice to the same extent. Consistently, rosiglitazone decreased also mitochondrial respiration rate at these supratherapeutic concentrations in both homogenates [62].

We then determined the effects of rosiglitazone on both mitochondrial and cytosolic rate-limiting enzymes controlling ATP synthesis. When incubated with fresh tissue homogenate or isolated mitochondria for 60 min, rosiglitazone at 1 and 3 μM did not affect the activities of cytosolic and mitochondrial enzymes tested as compared with vehicle treatment. At the supratherapeutic concentrations of 10 and 30 μM, however, rosiglitazone decreased the activities of myocardial mitochondrial complexes I and IV in both PPAR$\gamma^{-/-}$ and PPAR$\gamma^{+/+}$ mice to the same extent, but did not alter the activities of other mitochondrial enzymes citrate synthase, creatine kinase, Complexes II, III, V and cytosolic enzymes phosphofructokinase, lactate dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase [62]. These results indicate that the higher concentrations of rosiglitazone caused myocardial energy deficiency and mitochondrial dysfunction in the cardiomyocytes in a PPARγ-independent manner. Consistent with our study, Brunmair et al. reported that 10–100 μM TZDs rosiglitazone, troglitazone and pioglitazone inhibited mitochondrial complex I activity, respiratory control and glucose oxidation in the rat liver and skeletal muscles [63]; Rachek et al. found that troglitazone induced mitochondrial dysfunction and cell death in human hepatocytes [64]; results from Scatena et al. also suggested that TZDs induced a non-PPARγ-mediated effect: mitochondrial respiratory chain dysfunction [65].

The PPARγ-independence of rosiglitazone-induced energy deficiency and mitochondrial dysfunction are also supported by the following evidences: (1) Treatment with PPARγ agonist medium-chain triglyceride decanoic acid improved mitochondrial function as evidenced by increases in mitochondrial number, activities of mitochondrial enzyme citrate synthase, complex I, and catalase [66], whereas treatment with PPARγ agonist rosiglitazone induced mitochondrial dysfunction, suggesting rosiglitazone likely induces the mitochondrial dysfunction via PPARγ-independent mechanism [62]. (2) PPARγ-dependent effects are based upon altered transcription of genes involved in energy metabolism and usually require hours

to days to take into effect. The myocardial energy deficiency was observed after short time (30–60 min) exposure to rosiglitazone in our study [62]. Such an acute treatment generally does not allow gene expression to change after transcriptional activation of PPARγ, indicating rosiglitazone likely induced myocardial energy deficiency via PPARγ-independent mechanism.

To rule out the possibility that rosiglitazone caused myocardial energy deficiency and mitochondrial dysfunction through activation of PPARγ in other cardiac cells including fibroblast, smooth muscle cells and endothelial cells, we examined the effects of GW9662, a specific PPARγ antagonist on the detrimental actions of rosiglitazone on myocardial energy metabolism and mitochondrial function. We found that perfusion of hearts from C57BL/6 mice with 10 μM GW9662 for 60 min affected neither total ATP content, nor ATP/ADP ratio, nor energy charge. This antagonist did not reverse the decreases in total ATP content, ATP/ADP ratio and energy charge induced by rosiglitazone at 10 μM in those hearts. Furthermore, 10 μM GW9662 showed no effects on the oxidation rates of glucose and palmitate, mitochondrial respiration rate, or the activities of mitochondrial complexes I and IV, it did not antagonize the downregulations of those parameters by rosiglitazone at the supratherapeutic concentration of 10 μM, either [62]. Additionally, treatments with rosiglitazone at the supratherapeutic concentrations of 10 and 30 μM for 60 min significantly decreased intracellular ATP content in cultured mouse cardiomyocytes. In contrast, treatment with 10 μM rosiglitazone showed no effect on intracellular ATP content in cultured mouse cardiac fibroblasts, treatment with 30 μM rosiglitazone only slightly decreased intracellular ATP content in these fibroblasts. Interestingly, pretreatment with 30 μM GW9662 did not prevent the decreases in intracellular ATP content induced by rosiglitazone in these cultured cardiomyocytes or cardiac fibroblasts [62]. These results further support that rosiglitazone induces myocardial energy deficiency via PPARγ-independent mechanism.

To maintain energy homeostasis, the capacities of ATP synthesis by mitochondrial oxidative phosphorylation, glycolysis, and phosphotransferase (i.e., creatine kinase, CK) reactions must match the demand for ATP utilization by the sarcomere, ion pumps, etc. [67]. Therefore, increased ATP utilization and decreased ATP synthesis, singly or in combination, can cause energy deficiency. The free energy of ATP hydrolysis ΔGATP decreased following rosiglitazone treatment. Furthermore, heart mechanical work (assessed by rate pressure product, an indirect index of calcium cycling, metabolic demand, and ATP utilization) also decreased following acute treatment with rosiglitazone at 10–30 μM. These results suggest that decreased ATP synthesis may be responsible for myocardial energy deficiency induced by rosiglitazone. The main pathways for ATP synthesis in hearts are glycolysis, phosphoryltransfer reactions, and substrate oxidative phosphorylation. Rosiglitazone showed no effect on glycolytic rate-limiting enzymes and the product of CK activity and total creatine content [62], indicating that neither glycolysis nor phosphoryltransfer reaction is likely to be involved in rosiglitazone-induced myocardial energy deficiency.

The inhibition of complex I by rosiglitazone caused impaired oxidation of NADH and in turn decreased NAD content. As a result, NADH/NAD ratio increased. The impaired oxidation of NADH leads to decreased substrate oxidation and in turn decreased ATP synthesis. Complex

IV acts as the terminus of mitochondrial electron transport by accepting four electrons to reduce a single oxygen molecule. The reaction is coupled with the transfer of four protons across the mitochondrial membrane, driving ATP synthesis. Thus, the inhibition of both complexes I and IV by rosiglitazone reduces ATP synthesis, which manifests as the myocardial energy deficiency induced by rosiglitazone.

5. Rosiglitazone induces myocardial mitochondrial oxidative stress via PPARγ-independent mechanism

To assess the in vitro effects of rosiglitazone on redox homeostasis, we determined enzyme (NADPH oxidase, xanthine oxidase and mitochondrial complexes I and III)-dependent reactive oxygen species (ROS) O_2^- production, the capacity of ROS elimination systems including superoxide dismutase (SOD), reduced glutathione (GSH), glutathione peroxidase and catalase, and biomarkers malondialdehyde (MDA), protein carbonyl and 8-hydroxy-2′-deoxyguanosine (8HOdG) of oxidative damage to lipids, proteins and DNAs, respectively, in isolated mitochondria and nuclei. We found that at 1 and 3 μM, rosiglitazone showed no effects on any of the aforementioned parameters. At 10 and 30 μM, however, rosiglitazone increased mitochondrial complexes I- and III-dependent O_2^- production, decreased the level of mitochondrial GSH and SOD activity, and increased the levels of mitochondrial MDA, protein carbonyl and 8-OHdG [62]. Interestingly, pretreatment with 30 μM GW9662 did not prevent rosiglitazone-induced changes in the above redox parameters. Furthermore, even at the supratherapeutic concentrations of 10 and 30 μM, rosiglitazone did not affect the activities of catalase and glutathione peroxidase, and changed neither the level of nuclear protein carbonyl nor the level of nuclear 8-OHdG [62]. Similar to our study, rosiglitazone at 50 and 60 μM induced apoptosis via oxidative stress in cultured H9c2 cells [68].

We also assessed the acute effects of rosiglitazone on mitochondrial oxidative stress in vivo. At 1 mg/kg, injection of rosiglitazone into mouse tail vein showed no effect on the levels of myocardial mitochondrial MDA, protein carbonyl and 8-OHdG. At 10 mg/kg, however, rosiglitazone increased the levels of these mitochondrial oxidative stress markers. Importantly, injection of antioxidant N-acetyl-L-cysteine 600 mg/kg into mouse tail vein prevented the above rosiglitazone-induced changes of mitochondrial oxidative stress markers in vivo [62]. Furthermore, intravenous injection of GW9662 at 1 mg/kg, previously demonstrated to interact selectively with PPARγ, acting as a potent and full PPARγ antagonist, did not prevent 10 mg/kg rosiglitazone induced myocardial oxidative stress [62]. Taken together, our in vitro and in vivo data support that rosiglitazone induces myocardial mitochondrial oxidative stress via PPARγ-independent mechanism, possibly by decreasing mitochondrial ROS-scavenging capacity.

6. Rosiglitazone causes cardiac dysfunction via PPARγ-independent mechanism

Normal cardiac contractile function requires energy homeostasis. As we found that rosiglitazone caused energy deficiency, we therefore further determined the effects of rosiglitazone

on cardiac function. In ex vivo Langendorff-perfused hearts, treatment with rosiglitazone at 1 and 3 μM for 24–30 min showed no obvious effects on cardiac systolic function as assessed by left ventricular systolic pressure (LVSP) and the rate of tension development (+dP/dt). Treatment with rosiglitazone at 10 and 30 μM for 24–30 min, however, decreased LVSP and + dP/dt in hearts from C57BL/6, PPARγ−/− and PPARγ+/+ mice, indicating acute treatment with rosiglitazone at the supratherapeutic concentrations causes cardiac systolic dysfunction [62]. Similarly, treatment with rosiglitazone at 1 and 3 μM for 24–30 min showed no obvious effects on cardiac diastolic function as assessed by left ventricular end diastolic pressure (EDP) and the rate of relaxation (–dP/dt). Treatment with rosiglitazone at 10 and 30 μM for 24–30 min, however, increased EDP and decreased –dP/dt in all hearts from the above three genotypes, indicating acute treatment with rosiglitazone at the supratherapeutic concentrations also causes cardiac diastolic dysfunction [62]. Interestingly, rosiglitazone-induced cardiac dysfunction was not distinguishable among C57BL/6, PPARγ−/− and PPARγ+/+ mice, indicating acute rosiglitazone treatment caused cardiac dysfunction independently of cardiomyocyte PPARγ [62]. Additionally, treatment of hearts with 10 μM GW9662 for 60 min, affected neither cardiac function, nor rosiglitazone-induced cardiac dysfunction. In contrast, treatment of hearts with 20 mM N-acetyl-L-cysteine (NAC) for 60 min did not affect baseline cardiac function, but prevented cardiac dysfunction induced by rosiglitazone at the supratherapeutic concentration of 10 μM [62]. These data further support that rosiglitazone induced cardiac dysfunction via a mechanism related to oxidative stress and independent of PPARγ.

We also evaluated the side effects of rosiglitazone on cardiac function in vivo setting by using echocardiography. Injection of rosiglitazone at the dose of 1 mg/kg into mouse tail vein showed no effect on cardiac function as assessed by fraction shorting [29] and ejection fraction EF). At 10 mg/kg, however, rosiglitazone decreased FS and EF, indicating rosiglitazone caused cardiac dysfunction at a higher dose. NAC at 600 mg/kg alone showed no effect on cardiac function. In combination with 10 mg/kg rosiglitazone, however, this antioxidant prevented rosiglitazone-induced cardiac dysfunction. In contrast, intravenous injection of PPARγ selective antagonist GW9662 at 1 mg/kg did not prevent 10 mg/kg rosiglitazone-induced cardiac dysfunction [62]. These in vivo studies also support that rosiglitazone induces cardiac dysfunction via a mechanism related to oxidative stress and independent of PPARγ.

7. TZDs induce cardiac hypertrophy via PPARγ-independent mechanism

TZDs are expected to inhibit cardiomyocyte growth in vitro and in pressure overload models via activation of PPARγ. Paradoxically, TZDs have also been reported to induce cardiac hypertrophy in mice, rats and dogs [69, 70]. This side effect may occur because TZDs expand blood volume. However, an essential question is whether or not this effect is directly attributable to cardiac PPARγ activation. Treatment with TZD rosiglitazone 10 mg/kg per day for 4 weeks induced cardiac hypertrophy in both PPARγ−/− and PPARγ+/+ mice. Rosiglitazone treatment increased cardiac phosphorylation of p38 mitogen-activated protein kinase (p38-MAPK), a MAPK pathway essential for cardiac hypertrophy, in PPARγ−/− mice. The effect of rosiglitazone on p38-MAPK persisted in PPARγ−/− mouse hearts indicated that activation

of p38-MAPK by TZDs is independent of cardiomyocyte PPARγ [70]. Furthermore, phosphorylation of c-Jun N-terminal kinases was not affected by rosiglitazone or cardiomyocyte PPARγ deletion. Surprisingly, despite hypertrophy, AKT phosphorylation was suppressed in PPARγ−/− mouse hearts [70]. These data demonstrate that cardiomyocyte PPARγ suppresses cardiac growth and embryonic gene expression and inhibits nuclear factor κB activity in vivo, and that rosiglitazone causes cardiac hypertrophy at least partially independent of PPAR-γ in cardiomyocytes [70].

8. TZDs increase risks of heart failure

Congestive heart failure (CHF) is a major complication of diabetes and occurs as a result of both atherosclerotic coronary disease and non-ischemic diabetic cardiomyopathy. TZDs improve glycemic control and afford beneficial effects on many markers of cardiovascular risk including blood pressure, waist to hip ratio, HDL levels, endothelial reactivity, C-reactive protein, fibrinolysis, and microalbuminuria by improving peripheral insulin sensitivity. These antidiabetic agents, however, have been reported to worsen the existing CHF or precipitate new-onset failure in several reviews and meta-analyses of placebo-controlled randomized clinical trials (RCTs).

Bolen et al. found that the risk for CHF was higher with TZDs as either monotherapy or combination therapy than with metformin or sulfonylureas, with a range of 0.8–3.6% for TZDS and 0–2.6% for non-TZDs [4]. Lago et al. found an increased risk of CHF in use of TZDs in patients with diabetes and prediabetes compared with placebo and active-controls: relative risk 1.72, 95% confidence interval (CI) 1.21–2.42. The overall event rate for CHF with TZDs was 2.3% and with the comparison drugs 1.4% [14]. Singh et al. reported that the relative risk of CHF in use of rosiglitazone in patients with diabetes or prediabetes compared with various other antidiabetic drugs was 2.09 (95% CI 1.52–2.88) [17]. They also examined onset of CHF in both pioglitazone and rosiglitazone compared with placebo in three randomized controlled trials with subjects with either type 2 diabetes or prediabetes. The odds ratio (OR) for all heart failure adverse events was 2.10 (95% CI 1.08–4.08). Four observational studies produced an OR 1.55 (95% CI 1.33–1.80). These authors also examined case reports, including 162 case subjects with 99 analyzable cases. Among these cases, the median time to onset of CHF was 24 weeks, although failure could occur early and did not appear to relate to dosage. CHF was not limited to the elderly; 26% of cases were in subjects less than 60 years of age [71]. Hernandez et al. found that the TZD therapy was significantly and consistently associated with a higher risk of CHF: TZDs 360/6807 [5.3%] versus placebo 234/6328 [3.7%], OR 1.59; 95% CI 1.34–1.89; $p < 0.00001$. The risk of CHF was higher with rosiglitazone than with pioglitazone (OR 2.73; 95% CI 1.46–5.10) versus (OR 1.51; 95% CI 1.26–1.81; $p = 0.06$). Rosiglitazone and pioglitazone were associated with a similar risk of serious/severe CHF (OR 1.47; 95% CI 1.16–1.87; $p = 0.002$). The use of TZDs was also associated with edema (OR 2.04; 95% CI 1.85–2.26; $p < 0.00001$) [72]. The above increased risk of CHF was largely confirmed in other meta-analyses: the use of rosiglitazone for >4 weeks in 132 trials involving 41,743 patients with or without T2DM was associated with a 69% higher relative risk of serious CHF [73]; and

the combined short- and long-term use of pioglitazone in 19 RCTs involving 16,390 patients with T2DM found a 41% higher relative risk of serious CHF [74]. Another meta-analysis of 26 RCTs found 126% higher odds of peripheral edema in 15,332 diabetics with short- and long-term use of TZDs [75].

One of the potential mechanisms responsible for increased risk in CHF with TZD treatment may be the fluid accumulation observed in large-scale studies on antidiabetic medications [74–76]. In spite of a weak beneficial effect on blood pressure [77], volume overload beyond a certain threshold induced by TZDs increases the myocardial energy demand of the left ventricular and triggers metabolic disorder. As a compensatory mechanism, the contractile function of myocardia is temporarily restored via cardiac hypertrophy, overtaking the growing amount of mitochondrial respiration and ATP production gradually.

In susceptible individuals, these pathophysiological responses likely explain why rosiglitazone precipitate clinical heart failure, and why ischemic events are easily provoked. Notably, the sodium-retentive actions of rosiglitazone within the renal tubules are dose and duration-dependent and insulin-independent, accordingly, it is likely that concurrent treatment with insulin and rosiglitazone mutually reinforces the risk of each agent, thus markedly increases the possibility of worsening heart failure. The reasons for fluid retention and peripheral edema with TZD use are not fully understood and are likely to be multifactorial. One possibility is the reduction in renal excretion of sodium and an increase in sodium and free water retention. Whether these actions are PPARγ-dependent or not warrants further study.

The other potential mechanism responsible for increased risk in CHF with TZDs treatment may be related to their direct adverse effects on myocardial energy deficiency, mitochondrial function and cardiac function observed in our previous study [62]. It is well known that altered energy metabolism and cardiac dysfunction are common features of heart failure resulted from different causes, including diabetes. We demonstrated that rosiglitazone induced myocardial energy deficiency, mitochondrial dysfunction and cardiac dysfunction in perfused mouse hearts at the supratherapeutic concentrations of 10 and 30 μM and induced cardiac dysfunction in vivo at a high dose of 10 mg/kg [62]. TZDs might be accumulated over a longer period of time in the cell or their effects are in some other way "cumulative" in some patients who need increased doses due to the tolerance during a long period of therapeutic time, and in diabetic patients with renal dysfunction. Therefore, it is likely that TZDs increase the risk of heart failure in T2DM patients through their PPARγ-independent adverse effects on the heart.

9. TZDs increase risks of myocardial infarction

Muraglitazar, an investigational dual PPARα and PPARγ agonist, was the first TZD agent halted because of increased adverse cardiovascular events, including myocardial infarction, transient ischemic attack, and stroke, during phase 2 and 3 trials [78]. In 2007, Nissen and Wolski performed the first large meta-analysis of 42 trials involving 27,847 patients with

randomized control group not receiving rosiglitazone and found that rosiglitazone was associated with a significant increase in the risk of myocardial infarction (OR 1.43, 95% CI 1.03–1.98, P = 0.03) and with an increase in the risk of cardiovascular death (OR 1.64, 95% CI 0.98–2.74, P = 0.06) [16]. In 2010, Nissen and Wolski published an update including 56 trials with 35,531 randomized patients: 19,509 who received rosiglitazone and 16,022 who received control therapy. They continued to demonstrate that rosiglitazone therapy significantly increased the risk of myocardial infarction (OR 1.28, 95% CI 1.02–1.63, P = 0.04) [79]. Consistent with above analyses, Ontario study [80] and Taiwan study [81] also reported the increased risks in both myocardial infarction and cardiovascular death following the treatment with rosiglitazone. Several other meta-analyses by Psaty and Furberg, GlaxoSmithKline, U.S. FDA and Singh et al. found the increased risks in myocardial infarction but uncertainty in cardiovascular death in patients with rosiglitazone treatment [17, 82, 83], whereas meta-analysis by Shuster et al. reported the increased risk in cardiovascular death but uncertainty in myocardial infarction in subjects treated with rosiglitazone [84].

In contrast, the meta-analysis by Diamond et al. and Lago et al. reported that rosiglitazone was not associated with an increase in the risk of myocardial infarction and cardiovascular death [14, 85].

These discrepancies can be ascribed to the inconsistencies in trial design, eligibility, follow-up, sample size, analytical methodology, and endpoint criteria among analyses and studies.

The mechanisms responsible for increased risks in myocardial infarction and cardiovascular death related to TZDs are not fully characterized. Several contributing factors are possible: first, the reduction in hemoglobin. TZDs, including rosiglitazone, may produce a modest reduction in the hemoglobin level. In susceptible patients, a reduced hemoglobin level may result in increased physiological stress, thereby provoking myocardial ischemia [16]. The second is adverse effects on serum lipids. TZDs may produce detrimental influences on serum lipids. Rosiglitazone increased low-density lipoprotein cholesterol (LDL-C) concentration of 18.6% among T2DM patients treated for 26 weeks with an 8-mg daily dose via increasing serum paraoxonase activity, which protects LDL-C against lipid peroxidation. This TZD also significantly increased triglyceride levels in 50 patients who were given at 4 mg/day for 3 months in addition to their usual treatment compared to baseline levels [86, 87]. Higher LDL-C level was consistently and independently associated with higher incidences of major adverse cardiovascular events after controlling for conventional risk factors [88]. Third, overload of intravascular volume. TZDs may induce fluid retention and peripheral edema likely via the reduction in renal excretion of sodium and an increase in sodium and free water retention [13]. The volume overload increases stress on the left ventricular wall, a factor that determines myocardial oxygen demand. In susceptible patients, an increase in myocardial oxygen demand could theoretically provoke ischemic events.

10. Summary

There are two TZDs approved for prescription use in the United States: rosiglitazone maleate (Avandia) and pioglitazone hydrochloride (Actos). Both have been widely used to treat adult patients with T2DM, either as monotherapy or in combination with insulin, metformin,

or sulfonylurea when diet, exercise, and a single agent does not result in adequate glycemic control. The mechanisms of action of TZDs in lowering plasma glucose among patients with T2DM are thought to include the following: increase insulin sensitivity, decrease endogenous glucose production and postprandial gluconeogenesis, increase fasting and postprandial glucose clearance, and have beneficial effects on beta-cell function. The glycemic effects of these agents are thought to be mediated by binding to PPARγ (**Figure 1**).

Their efficacy and beneficial effects, however, are shadowed by the increased risks of cardiovascular adverse events. Evidences are accumulating that TZDs, particularly rosiglitazone, cause cardiotoxicity including myocardial energy deficiency, mitochondrial dysfunction, and oxidative stress with concomitant cardiac dysfunction in ex vivo perfused hearts. TZDs may also cause cardiac hypertrophy in whole animal model. Additionally, TZDs increase the risks of heart failure and myocardial infarction in patients with T2DM. Understanding whether the cardiotoxicity induced by TZDs is PPARγ independent or not is an important issue for designing more specific PPARγ agonists with fewer side effects. TZDs also have affinity to numerous non-PPARγ targets in mitochondria, cytosol and cytoplasm, including

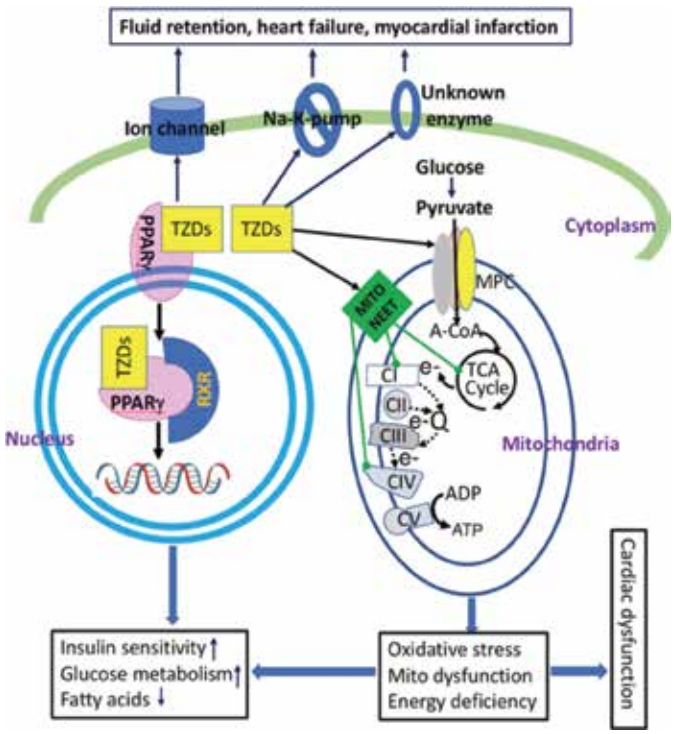

Figure 1. PPARγ-dependent (on-target) and -independent (off-target) effects of thiazolidinediones (TZDs). TZDs produce on-target effects by binding to nucleus PPARγ, increasing insulin sensitivity and glucose oxidation and contributing to efficacy of the drugs. They produce off-target effects by binding to numerous non-PPARγ targets including MitoNEET, mitochondrial pyruvate carrier (MCP), dehydrogenases involved in TCA cycle and electron transport chain complexes, cytoplasmic ion channels, Na-K-pump and other unknown enzymes. Off-target effects potentially increase cardiotoxicity including mitochondrial (Mito) dysfunction, oxidative stress and myocardial energy deficiency, fluid retention, congestive heart failure and myocardial infarction. Paradoxically, Mito dysfunction and energy deficiency may also stimulate insulin sensitivity and glucose uptake in the heart and indirectly contributing to efficacy of the drugs. A-CoA, acetyl-coenzyme A; TCA, tricarboxylic acid; e–, electron; CI, CII, CIII, CIV and CV, mitochondrial respiratory chain complexes I, II, III, IV and V, respectively; Q, coenzyme Q.

MitoNEET, mitochondrial pyruvate carrier (MCP), dehydrogenases involved in TCA cycle and electron transport, cytoplasmic ion channels, Na-K-pump and other unknown enzymes. By binding to these non-PPARγ targets, TZDs produce off-target effects and potentially increase cardiotoxicity including mitochondrial dysfunction, oxidative stress and myocardial energy deficiency, fluid retention, congestive heart failure and myocardial infarction. Paradoxically, mitochondrial dysfunction and energy deficiency may also stimulate insulin sensitivity and glucose uptake in the heart and indirectly contributing to efficacy of TZDs. Therefore, TZDs may produce antidiabetic effects via both PPARγ-dependent and PPARγ-independent mechanisms, and they may induce cardiotoxicity solely via PPARγ-independent mechanism (**Figure 1**). This chapter also raised concerns that the use of TZDs may lead to a significant increase in adverse cardiovascular effects. The benefit/risk profile of TZDs should be considered when treating diabetic patients with or without prior cardiovascular diseases.

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Conflict of interest

Authors declare no conflict of interest.

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Chapter 5

Cocaine Cardiac Toxicity: Revisited

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Additional information is available at the end of the chapter

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Abstract

Cocaine is a potent stimulant which affects cardiovascular system severely. The mechanism of cardiac toxicity depends on multiple factors. Cocaine increases sympathetic stimulation and causes excess catecholamine secretion. Besides, its indirect sympathomimetic effect also directly exerts cardiotoxic effect by different cellular, molecular, and ionic mechanisms, resulting in acute or chronic cardiovascular impairment. Cardiac arrhythmia and acute myocardial ischemia or infarction is the most common cause of cocaine-induced sudden cardiac death. Chronic cocaine abuse can develop sustained hypertension or myocarditis or cardiomyopathy leading to depressed left ventricular function. Therapy for cocaine induced cardiac toxicity generally includes use of benzodiazepine agents, nitric oxide mediated vasodilators, alpha blockers and even calcium channel blockers. Beta blockers are relatively contraindicated in acute settings of cocaine cardiovascular toxicity. Hypersensitivity reaction to cocaine is often manifested by infiltration of eosinophilic or mononuclear cells without myocardial cell damage. Vascular dissection, endocarditis, and tricuspid valvular abnormalities are some less frequent manifestations in cocaine-induced cardiac toxicity.

Keywords: cocaine, ischemia, atherosclerosis, cocaine cardiomyopathy, cocaine myocarditis

1. Introduction

Cocaine is a potent natural alkaloid derived from the leaves of South American Coca tree (*Erythroxylum coca*). It was known as one of the oldest stimulants that were used by the ancient Inca in the Andres Mountain for simultaneous acceleration of their heart and respiratory rate to counter the effect of low pressure of the mountain air in the 3000 B.C. [1]. The

drug was first extracted in 1859 by German alchemist Albert Niemen from the coca leaves. In the past, cocaine was considered a medicine. Austrian psychoanalyst Sigmund Freud used it as a medicine for sexual impotence and depression. However, once hailed as a magical drug, it is currently known as the most addictive substance on the earth [2].

Cocaine is the second most popular drug addiction after marijuana. According to National Survey of Drug Use and Health (NSDUH), cocaine abuse is most common in the young population between 18 and 25 years in the United States [3]. The data from 2011 Drug Abuse and Warning Network (DAWN) cocaine abuse involve 40% of the total cases of the drug misuse cases in the emergency department [4].

Pure cocaine can be abused by chewing coca leaves (*Erythroxylum coca*). However, the more popular way of cocaine abuse is smoking or snorting. On the street, the drug is available in hydrochloride salt form or as free base form. In order to prepare hydrochloride salt, pure white crystal-like cocaine powder is treated with acid solvent. Hydrochloride salt is most commonly snorted or taken through intravenous route as the salt is water soluble. On the other hand, free base cocaine is produced by mixing with ammonia and ether as solvent and then dried to a powder form. Free base is more pure and more addictive when it is smoked or snorted. Crack cocaine is the most commonly used form of free base form of cocaine. Crack cocaine is synthesized by heating cocaine hydrochloride and baking soda solution to form a hard rock like cocaine base. It crackles when smoked. Drug dealers usually cut cocaine mixing with some adulterants. These additive materials are added to the cocaine to enhance its effect as well as increasing the amount. Glucose, mannitol, laundry detergents, laxatives, local anesthetic like lignocaine, strychnine, amphetamine are very popular additives while making cocaine. "Speed ball" is a popular addictive substance where heroin is added with cocaine [5].

The drug can be absorbed through mucous membranes. The peak effect of cocaine is very fast when it is taken by inhalation or intravenous route. However, nasal insufflation of drug exerts a slower action as the peak serum concentration is reached after 30–60 minute. Cocaine itself inhibits its absorption due to its vasoconstriction effect [5]. Cocaine is rapidly metabolized in the body. Its serum half-life is 45–90 minute. Cocaine can be detected for several hours in the blood and urine sample after its use. It is metabolized into two major metabolites: benzoylecgonine and ecgonine methyl esters, which are capable of producing hypertension. Norcocaine is another minor metabolite, also known for its vasoconstrictive effects. Cocaine metabolites can be detected in the blood and urine sample even after 4 days of cocaine use. Hair analysis is a sensitive marker of detection of cocaine and its metabolites for chronic cocaine abuse [6].

2. Pathogenesis of cocaine cardiac toxicity

Cocaine abuse affects multiple organ systems including the neurological, cardiovascular, immune, and hematological systems simultaneously due to its complex pharmacological action. It binds to membrane-bound proteins, which include transporters, receptors, and voltage-gated ion channels.

- Generalized sympathomimetic activity

Sympathomimetic effects of cocaine take place when it binds to three monoamine transporters of the nerve terminals: the serotonin transporter (SERT), the dopamine transporter (DAT), and the norepinephrine transporter (NET). Cocaine can bind also two neurotransmitter receptors: muscarinic and sigma receptors. Interaction with monoamine transporters along with neurotransmitter receptors lead to a cascade of cocaine-induced stimulation of central sympathetic outflow. Thus, it inhibits reuptake of the neurotransmitters from the extracellular space. The increased concentration of the neurotransmitter in the synapse causes enhanced postsynaptic transmission. As a result of sympathetic stimulation, cocaine causes vasoconstriction, hypertension, and increased myocardial oxygen demand [7] (**Figure 2**).

2.1. Cardiac arrhythmia

Pathophysiology of the cocaine cardiac toxicity further includes inducing cardiac arrhythmia, which is one of the very common causes of death in the emergency department. Cocaine-abuse-related cardiac arrhythmia is considered having multifactorial underlying causes.

- Increase in the concentration of catecholamine and generalized sympathomimetic effect is one of the causes of genesis of cardiac arrhythmia triggered by cocaine. Further cocaine-induced myocardial ischemia or infarction provides a potential substrate for arrhythmia [8] (**Figure 2**).
- Blockade of voltage-gated Na^+ and K^+ channels

Cocaine is capable of binding to cardiac sodium, potassium calcium channels at clinically significant concentration. Inactivation of voltage-gated sodium and potassium channel by cocaine leads to intracardiac slowing of conduction and myocardial suppression. In Guinea pig model, cocaine blocks ventricular fast voltage-gated Na^+ channels in a dose-dependent and reversible manner [9, 10]. These result in decreased myocardial contractility, cardiac arrhythmia, and decreased left ventricular ejection fraction. Prolongation of the cardiac ventricular depolarization period leads to reentrant arrhythmia. Bruguda syndrome like electrocardiographic pattern can be secondary to mechanism of cocaine-related Na^+ channel blockade [11] (**Figure 1**).

Besides, cocaine on Na^+ ion channels also causes blockade of potassium and L-type Ca^+ channels. The potassium current is significantly diminished following the drug abuse. Cocaine blocks the human ether–a-go-go-related gene (h-ERG) channel to delay the rectifier K^+ current (I_{kr}) and thereby inhibits repolarization phase of the action potential. Prolongation of the action potential results in QT prolongation in habitual cocaine users [12].

At a higher serum concentration, cocaine binds to protein with lower affinity as the proteins with high affinity get saturated. At lower concentration, high-affinity monoamine transporters are inhibited. It binds to voltage-gated ion channels at a higher concentration as monoamine transporters get saturated. In animal model, progressive cocaine poisoning at higher serum concentration demonstrates cardiotoxicity along with CNS effect [7].

- Ca^+ homeostasis

Cocaine-induced binding and opening of L-type Ca^{+} channels causes increase in intracellular Ca^{+} ion concentration due to ionic influx in cardiomyocyte in experimental model. The drug promotes opening of L type Ca^{+2} channels and decreases closing rate. Consequently, intracellular Ca^{+} level rises and also provides additional reason for cardiac arrhythmia by secondary messenger pathway [13].

Calsequestrin is a major Ca^{2+} storage protein in sarcoplasmic reticulum of the cardiac and skeletal muscle cells. Cocaine is capable of disrupting the excitation contraction coupling through prevention of polymerization of calseqestrin and its calcium binding capacity in sarcoplasmic reticulum of cardiomyocyte. As a result, it reduces sarcoplasmic reticulum Ca^{2+} storage and cytosolic release of Ca^{2+}, leading to cardiac arrhythmia [14] (**Figure 1**).

2.2. Myocyte damage

Cocaine causes myocardial damage by both direct and indirect pathway resulting in different morphological and histological abnormalities including contraction band necrosis, cardiomyopathy, myocardial fiber disarray, mononuclear cell infiltration, etc. [11] (**Figures 1** and **2**).

- Myocardial cell apoptosis

In animal model, cocaine is able to cause apoptosis of the myocardial cells in a dose- and time-dependent manner through mitochondrial pathway. It is found an increase of cytochrome c enzyme levels in the cytosol in comparison with its level in mitochondria in fetal rat myocardial cells exposed to different doses of cocaine for different time intervals. Cytosolic cytochrome c binds to apoptotic activating factor 1 (Apaaf 1) and d ATP. It produces activated caspase 9, which cleaves procaspase 3 to create active caspase 3. Cocaine is believed to cause apoptosis of the myocardial cells by increasing caspase protein activities in animal model. Furthermore, cocaine increases Bax protein levels and decreases Bcl 2 in vivo to increase caspase activity and mitochondrial cell death [11]. Myocardial cell apoptosis is also demonstrated by cocaine-induced rise of p38 MAPK (mitogen-activated protein kinase) and inhibition of cytoprotective extracellular signal regulated kinase [11]. Wang et al. proposed a role of TNF α in cocaine cardiotoxicity. Myocardial TNF α induces myocardial apoptosis through its action causing chronic heart failure in animal model [15].

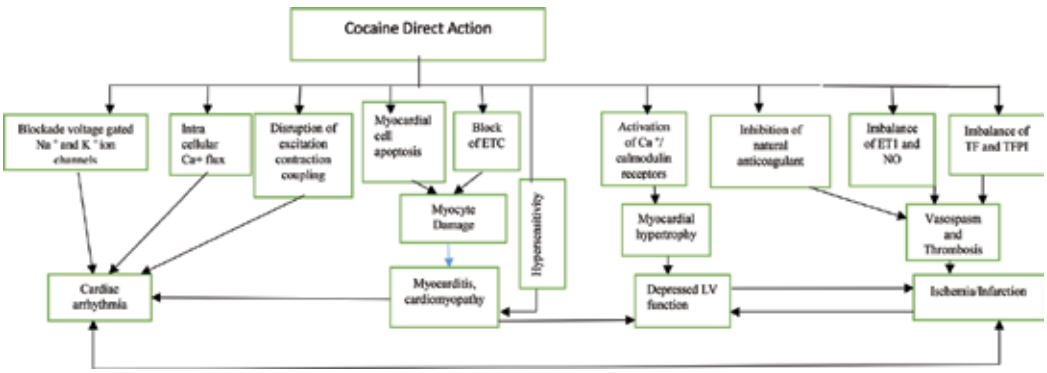

Figure 1. Schematic diagram of direct effect of cocaine in cardiotoxicity.

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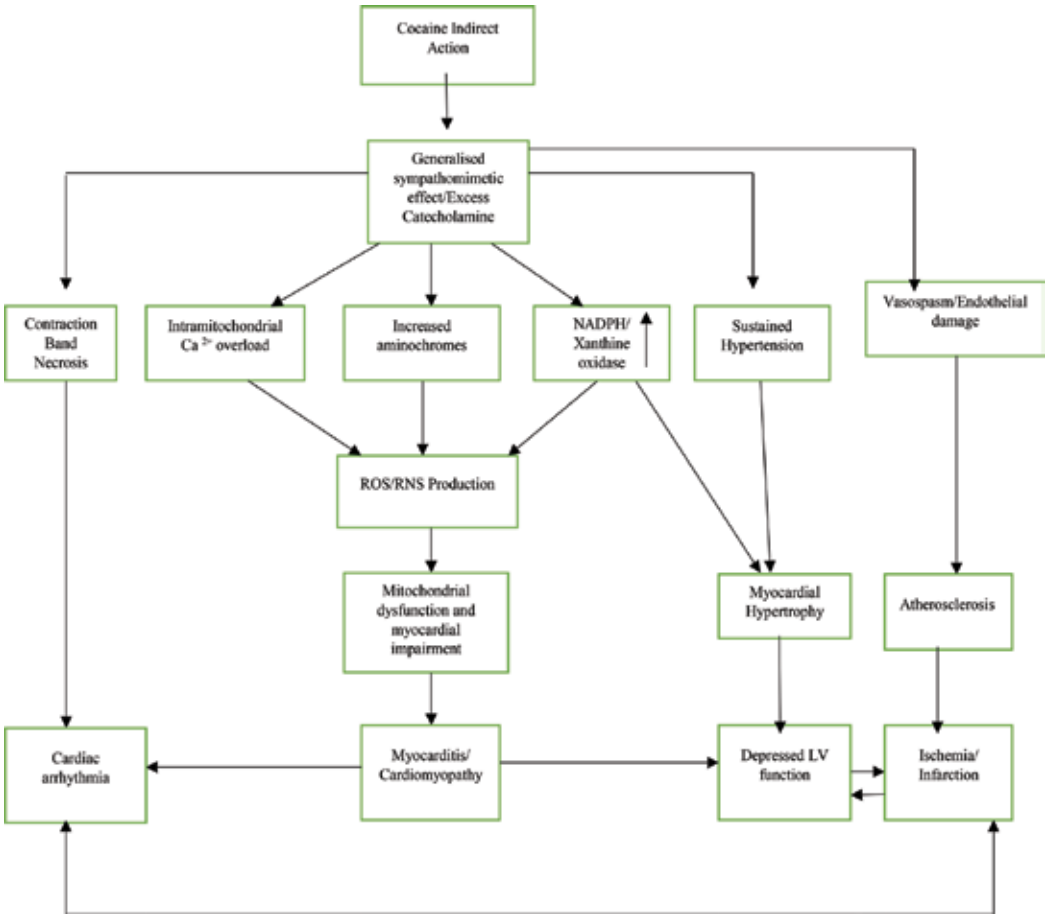

Figure 2. Schematic diagram of indirect effect of cocaine in cardiotoxicity.

- Mitochondrial dysfunction

Cocaine triggers indirect pathway of myocardial damage by production and accumulation of reactive oxidation species (ROS) and reactive nitrogen species (RNS) due to enzymatic or nonenzymatic catabolism of the cocaine-induced excess catecholamines, which causes mitochondrial damage and cell death.

Cocaine directly inhibits mitochondrial electron transport chain leading to decreased ATP production and formation of ROS and RNS. On the other hand, indirect release of high levels of circulatory catecholamines disrupts Ca^{2+} homeostasis. It results in a rise of intramitochondrial Ca^{2+} overload and depletion of ATP production. Moreover, catabolism of catecholamines produces different amino chromes (adrenochromes, dopachrome, and noradrenochrome), which are active sources of ROS. Further contribution of the mitochondrial ROS production is obtained from the increased NADPH oxidase and xanthine oxidase activity on high levels of catecholamines. Increased mitochondrial ROS and RNS cause mitochondrial dysfunction and subsequent cardiotoxicity [16].

2.3. Myocardial hypertrophy

It is developed from cocaine-induced sustained hypertension by sympathomimetic effect on the heart [17]. In addition, cocaine stimulates α 1 adrenoceptors to increase in total protein content along with β myosin heavy chain expression in the myocytes [18, 19]. Cocaine-induced production of ROS by stimulating α_1 adrenoceptors mediated by NADPH oxidase promotes hypertrophic growth ventricular myocytes [20, 21]. Finally, cocaine-induced direct activation of calcium/calmodulin receptors that culminates in increased phosphorylation of the ryanodine receptors leads to increased Ca^{+} release from sarcoplasmic reticulum and increase in protein formation in the myocytes [22] (**Figures 1** and **2**).

2.4. Effect on the blood vessels

- Atherosclerosis and vasospasm

Cocaine is known for developing premature atherosclerosis even in the young abuser (mean age 32 years) [23]. Cocaine and its metabolites induced sympathomimetic effect that leads to prolonged vasoconstriction (**Figure 2**). Endothelial damage secondary to vasoconstriction forms the basis of atherosclerosis. Increase of the histamine and heparin, secreted from increased mast cell, is considered to increase endothelial cell permeability [24]. Increased endothelial cell permeability secondary to cocaine and coco ethylene (formed following cocaine abuse along with alcohol intake) causes deposition of the low-density lipoprotein in the vasculature causing atherosclerosis [25].

Cocaine triggers endothelial production of endothelin 1 (ET 1). In animal study, ET 1 is shown to have vasoconstriction effect [26, 27]. Endothelium-derived nitric oxide (NO) is a potent vasodilator. The bioavailability and biological activity of NO is reduced due to reduced expression of endothelial NO synthase (e NOS) in presence of cocaine [28]. Cocaine in clinical concentration in abusers can cause significant imbalance between the concentration of the ET 1 and NO [11] (**Figure 1**).

- Thrombus formation

Kugelmas reported significant expression of the P-selectin protein on the platelet surface that activates alpha granules leading to formation of platelet thrombus. Intravenous administration of cocaine may cause significant expression of P selectin protein [29]. Hollander et al. in their study demonstrated cocaine-induced thromboxane release from activated platelets with a decrease in prostacyclin, an inhibitor of platelet aggregation [25].

Production of endogenous thrombosis and thrombolysis may be found through intranasal cocaine administration. It is associated with high level of plasminogen activating factor 1 (PAI 1) [30]. PAI 1 is released with α granules when platelet is activated. It contributes to thrombogenic activity by inhibiting fibrinolysis. Altered blood viscosity and increase in Von Willebrand factor, a glycoprotein secreted from the endothelial cells, also attribute to cocaine-mediated thrombogenesis [31].

Thrombus formation also results from the cocaine-induced increased expression of tissue factor (TF) and reduced expression of the tissue factor pathway inhibitor (TFPI) in the dysfunctional endothelial cells [32] (**Figure 1**).

Finally, cocaine inhibits formation of natural anticoagulant antithrombin III and protein C contributing to its prothrombotic property [25].

3. Pathology of cocaine cardiac toxicity

The morphological changes in the heart are attributed directly to cocaine toxicity as well as to indirect action of the excess catecholamine secretion. Apart from these, the hypothesis on myocardial changes can occur due to hypersentivity reaction to cocaine.

3.1. Contraction band necrosis

Excess catecholamine contributes significant changes in the myocardial fibers and that change is different from myocardial ischemia and infarction. The change or lesion is known as contraction band necrosis (CBN). It is also termed as myofibrillar degeneration and sometimes coagulative myocytolysis. CBN can be found in any circumstances related to catecholamine excess as well as in drowning, intracerebral hemorrhage, coronary artery occlusion sudden cardiac death, and in wide variety of drug overdose like amphetamine, MDMA, phenylpropanolamine, etc.

CBN is the earliest visible manifestation of excess catecholamine characterized by presence of individual necrotic myocardial cells surrounded by normal healthy myocells unlike ischemia where all myocells supplied by a single vessel are damaged. In addition, myofillaments are in register in ischemia unlike in CBN where myofillaments are fragmented forming eosinophillic clamps. CBN is a condition where myocytes are hypercontracted with sarcomeres of shorter length (less than 1.5 μm). Z lines get thickened and are often termed as "Contraction bands." However, CBN is also considered as ischemic change as it can be developed following coronary occlusion and then reperfusion of the ischemic cells. The underlying mechanism of formation of CBN is considered diverse; however, intracellular Ca^{+} overload mediated by catecholamine is largely responsible for such morphological alteration [33, 34].

3.2. Myocardial disarray

Cocaine abuse leads to degenerative and inflammatory changes of the myocardium. Myocardial disarray is characterized by oblique or perpendicular alignment of the adjacent myofibers and joined by short hypertrophied myobridges. It is often associated with CBN, interstitial edema, and focal myocardial fibrosis. CBN along with myocardial disarray, fibrosis can act as an anatomical substrate to trigger fatal cardiac arrhythmia causing sudden cardiac death [35].

3.3. Myocarditis and dilated cardiomyopathy

Virmani et al. in their autopsy study has confirmed cocaine-induced myocarditis is an important cause of death in cocaine abuse besides formation of CBN. Different cocaine preparations containing different adjuvants, different route of drug delivery, and chronicity of drug abuse could be the reason of different histopathological findings [36]. In cocaine-induced myocarditis, small foci of myocytes necrosis are scattered in different areas of the heart associated with infiltrations of lymphocytes. Under electron microscope, vacuolization of the sarcoplasmic reticulum and loss of myofibrils are found in the lesion. The mononuclear cellular infiltrations can be associated with various degrees of interstitial fibrosis. Biopsy specimen findings that include myocytes necrosis along with mononuclear cellular infiltration containing lymphocytes and monocytes secondary reaction to myocytes necrosis may be a result of acute cocaine-induced direct cardiotoxicity [37, 38]. Chronic cocaine abuse may lead to development of interstitial fibrosis and congestive heart failure [39].

However, mononuclear cell infiltrations as well as eosinophillic infiltration in the myocardium is also postulated as hypersensitivity reaction to cocaine or any contaminants along with cocaine. In such cases, the following features are characterized: (1) absence of myocytes necrosis, (2) absence of any myocardial hemorrhage, (3) lesions are of same age, and (4) not related to dose of the drug. Hypersensitive eosinophillic myocarditis is very often secondary to abuse of crack cocaine containing large amount of bicarbonate [33, 37].

Mere cellular infiltration in the myocardium does not indicate development of myocarditis. According to Dallas criteria, a lymphocytic infiltration without necrosis does not prove myocarditis [33].

Lymphocytic myocarditis serves also anatomic substrate for cardiac arrhythmia and ultimately develops a permanent dilated cardiomyopathy [37].

3.4. Myocardial hypertrophy

Cocaine user's heart weight found in autopsy studies is usually 10% heavier than mean weights predicted by a standard nomogram. ECG study also reaffirms the existence of ventricular hypertrophy in regular cocaine users. Hypertrophied heart includes hypertrophied myocytes, increased collagen deposition, perivascular fibrosis, and medial hypertrophy of small arterioles [17].

3.5. Myocardial infarction

Cocaine-induced vasospasm or vascular wall thickening causes arteriolar narrowing. In addition, its positive ionotropic and chronotropic effect leads to myocardial oxygen demand. Rise of heart rate and blood pressure after cocaine abuse also contributes to medial thickening of the intramyocardial vessels. Finally, atheromatous effect of cocaine contributes to significant narrowing of the coronary blood vessels, leading to acute myocardial ischemia and infarction. AMI with nonatherosclerotique coronary artery supports the hypothesis of coronary spasm and subsequently the thrombus formation with increased myocardial oxygen demand especially in acute drug intoxication setting [40].

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4. Clinical manifestations of cocaine cardiac toxicity

4.1. Myocardial ischemia and infarction

Myocardial ischemia develops following cocaine abuse due to:

- Increased sympathomimetic effect leading to rise in heart rate and myocardial oxygen demand
- Focal vasospasm of the coronary artery
- Increased atherosclerotic changes of the artery.

There is a multifold increased risk of acute MI during initial 1 hour after the use of cocaine use even in low-risk patients. Acute MI is manifested in 0.7–6% abusers with cocaine-associated chest pain. The incidence is much higher (25%) in younger people aged between 18 and 45 years old especially in those who are associated with other cardiac risk factors. Cocaine-associated chest pain is often substernal and pressure like associated with atypical presentations like pleuritic chest pain, nausea, palpitations, syncope, and vomiting. AMI reported is more among chronic abusers than first-time users. Most often, the drug is abused by smoking, intravenous, or intranasal route. Both Q and non-Q wave infarcts were found in different studies. About one-third cases of cocaine-related AMI are associated with normal anatomy of the coronary artery due to focal vasospasm of the artery or thrombolysis subsequent to blockade. However, the presence of thrombi or atherosclerosis is also evident in different autopsy studies related to cocaine-related death. Besides, myocardial infarction and myocardial ischemia without chest pain are often manifested with ST-T wave abnormalities on the ECG. This syndrome is often associated with normal epicardial coronary artery with marked thickening of the walls of the intramural coronary artery. Chronic users of cocaine are also susceptible to coronary vasospasm. Prinzmetal's angina like ST segment elevation is demonstrated in some abusers during first 2 weeks of withdrawal from cocaine [37, 41, 42].

- Cardiac biomarker

Cocaine-associated myocardial ischemia is not associated with elevation of CK-MB. However, elevated CK is demonstrated in 39% of the 49 cocaine users due to rhabdomyolysis, muscular trauma, or intramuscular injection. However, troponins are more specific cardiac biomarkers for cocaine users followed by CK-MB and CK, which is not specific [41].

Usually, acute ischemia or infarction develops within 12 hour of cocaine abuse. Weber et al. proposed a 12-hour observation period for cocaine abusers. High risk patients are identified by the following four parameters [43].

- ST segment depression or elevation of 1 mm or more that persisted for more than 1 minute
- elevation of troponin biomarkers
- recurrent chest pain
- hemodynamic instability

- Therapy consideration

Cocaine-induced ischemia or infarction can be treated with oxygen, aspirin to prevent thrombus formation, and nitroglycerine to revert coronary spasm. Nitroglycerine is able to relieve the chest pain in approximately half of the cocaine users. Nitroglycerine abolished Ach-induced vasoconstriction in long-term cocaine users. Intravenous benzodiazepine is used to prevent hypertension, tachycardia, academia, and hyperthermia. Benzodiazepine is also used to relieve the anxiety and chest pain in cocaine abusers. Phentolamine, an alpha receptor blocker, can be used in acute coronary syndrome especially in hypertensive emergencies. In a study on 45 patients, phentolamine is able to abolish successfully tachycardia, hypertension coronary artery diameter, and coronary sinus blood flow. Phentolamine is also useful for the treatment of cocaine-induced chest pain and ST segment elevation. Heparin, clopidogrel, and glycoprotein IIb/IIIa inhibitors have been used to lyse thrombus. Calcium channel blockers are considered second-line treatment for not responding to benzodiazepines or nitroglycerine. Calcium channel blockers improve heart rate, blood pressure, and myocardial contractility of the patients. All beta blockers should be avoided in acute setting in cocaine users.

Percutaneous coronary intervention is more desirable in cocaine using patients associated with myocardial infarction. Angiography can be important guidance to detect presence of thrombus and obstructive disease. Coronary thrombectomy along with glycoprotein IIb/IIIa inhibitors is advisable during PTCA in cocaine-associated ST segment elevation myocardial infarction. Stent thrombosis is important complication in continued and noncontinued cocaine users within approximately 8 months of antiplatelet therapy with bare metal and eluting stents [37, 40, 41].

4.2. Cardiac arrhythmia

Fatal cases of ventricular tachycardia, ventricular fibrillation, and sudden death related to cocaine abuse are reported frequently. However, cardiac arrhythmia can be manifested with or without myocardial ischemia or infarction resulting from the drug abuse. Cocaine prolongs QT interval and sometimes develops QT prolongation associated with ventricular tachycardia or Torsade de pointes [37]. In hypertrophied myocardium QT interval dispersion (difference between the QT length in the lead where it is maximum and QT length in the lead where it is minimum) of more than 80 ms, there is a loss of synchronized depolarization developing reentrant arrhythmia [17]. Cocaine-related Na^+ channel blockade replicates electrocardiographic pattern of Bruguda syndrome and is associated with ventricular fibrillation, polymorphic ventricular tachycardia, and sudden death in young patients with morphologically normal hearts [6].

- Therapy consideration

There is no specific treatment available for cardiac arrhythmia secondary to cocaine abuse. Antiarrhythmic agents class I and III can be avoided as they can further prolong the QT interval [44]. Benzodiazepines can be beneficial to manage supraventricular and ventricular tachycardia and tachyarrhythmia by inhibiting the drug's central stimulatory effect. In addition, it can reduce anxiety and agitation in cocaine abusers. Most atrial arrhythmia can also be responded to sedative drugs. The second line of drugs could be calcium channel blockers [6].

Early onset of cardiac arrhythmia following intake of the cocaine can be treated by sodium bicarbonate, which can reverse Na^+ channel blockade caused by cocaine and normalize the acid base balance. Ventricular arrhythmia manifested after several hours of cocaine intake is usually associated with myocardial ischemia and traditional treatment could be appropriate in such cases [6, 25].

4.3. Congestive heart failure

In long-term and acute cocaine intoxication, cocaine causes systolic dysfunction of the heart due to left ventricular dilation, decreased contractility, and increased end-diastolic pressure. Dilated cardiomyopathy is a very common complication of chronic cocaine abuse [41]. Left ventricular apical ballooning in Takotsubo cardiomyopathy of the heart is also reported in chronic cocaine abuse [44]. In acute cocaine intoxication, myocardial contractility and ejection fraction are reduced to increase end diastolic pressure and end systolic blood volume [45]. Chronic cocaine abuse leads to left ventricular hypertrophy and prolonged deceleration time [46].

- Therapy consideration

Cocaine-induced heart failure significantly improved following cessation of cocaine intake. However, it can be aggravated following resumption of cocaine abuse. Beta blockers are avoided in acute setting. In long-term cocaine abuse, cardiac transplantation is not beneficial [41].

5. Miscellaneous clinical manifestations

5.1. Dissection of the blood vessels

Dissection of aorta and coronary arteries is not very common, but it is found in "Crack" cocaine abuser due to severe hypertension and catecholamine release. Aortic medial disease and sustained severe hypertension are the usual risk factors. Aortic dissection is initiated by transverse tears in the aortic wall. In aortic dissection, tears usually extend through the intima and at least halfway through the media. However, in spontaneous coronary dissection, the dissection plane lies within media or between media and adventitia. In coronary artery dissection, eosinophillic periadventitial inflammation is commonly seen [33].

5.2. Endocarditis and valvular heart disease

Occasionally, in intravenous cocaine abuse, bacterial endocarditis and tricuspid valvular heart disease is reported in the literature. The association of the bacterial endocarditis development might be due to poor hygiene and frequent drug injection. However, the possibility of pathological effect of the cocaine including endothelial damage and thrombus formation cannot be ruled out [33, 41].

5.3. Sudden cardiac death

Sudden cardiac death secondary to cocaine abuse is rarely due to AMI. In most cases, cardiac arrhythmia is due to complex multifactorial consequence of cocaine cardiac toxicity [47].

Isolated several single case reports show that acute cocaine fatalities are found very often in cocaine body packers and body stuffers [48, 49]. Body packers smuggle by ingesting or inserting packets of cocaine or any illicit drugs in several body cavities or orifices to conceal them from law enforcement officials as they cross the international borders, and the drugs are subsequently retrieved in the country of arrival. Internal concealment of cocaine very often poses serious life-threatening conditions among body packers, such as cocaine intoxication due to rupture of the drug packets. The most serious consequence of such acute drug overdose is very often sudden cardiac arrest, cardiac arrhythmia or myocardial ischemia, and infarction [50].

Sudden cardiac death is also found in chronic cocaine users where patient's conditions are complicated by poor underlying cardiovascular conditions including coronary artery disease, myocarditis, cardiomyopathy, etc. [49].

- Alcohol

Cocaine abusers use alcohol along with drug to enhance the euphoric effect of the drug. Simultaneous alcohol use increases the chance of sudden cardiac death by many folds. It is found that alcohol increases serum concentration of the drug by 30% if alcohol is consumed prior to nasal inhalation of cocaine. However, this effect is not found following intravenous administration of cocaine or if it is taken before alcohol consumption. Bioavailability of the drug is increased following alcohol-induced nasal vasodilation. In addition, hepatic conversion of the cocaine into cocaethylene in presence of alcohol results in prolonged cardiac toxicity. Coco ethylene is known for increasing heart rate and dysrhythmogenic. The long lasting activity of the metabolite is responsible for increased chance of cocaine-induced sudden cardiac death in presence of alcohol [6, 51].

- Tobacco smoking

Combined tobacco smoking and cocaine abuse is responsible for diminution of the effective diameter of the diseased blood vessels. Vascular stenosis most commonly results in acute myocardial ischemia or infarction [6].

- HIV infection

In cocaine addicts with HIV infection positive, a cumulative effect of cocaine and HIV infection are found in cardiovascular system. It includes increased frequency of coronary wall and adventitial infiltration, thickening of the intramyocardial coronary arteries, and myocarditis [52].

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Section 3

Clinical Reasearch: Prevention, Prediciton and Treatment of Cardiotoxicities

Chapter 6

Concurrent Administration of Trastuzumab and Anthracycline for Breast Cancer Treatment: An Unassailable Contraindication?

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Additional information is available at the end of the chapter

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Abstract

Anthracyclines have a severe adverse effect in cardiac function. Same here, trastuzumab has cardiotoxicity even in single use and also should lead to exacerbation of anthracycline-induced cardiotoxicity. Concurrent administration of anthracycline and trastuzumab is dangerous, but sequential administration is also dangerous. We should carefully design trastuzumab-containing regimens based on anthracycline dosing, regardless of whether it is concurrent or sequential. Contraindication of concurrent use of anthracyclines and trastuzumab has distracted us from its potential efficacy as well as from the inherent danger of anthracyclines together with trastuzumab. Avoidance of concurrent dosing is insufficient. As anthracyclines and trastuzumab are essential agents for HER2-positive breast cancer, and so, we must continue to address this issue from both safety and efficacy aspects.

Keywords: anthracycline, trastuzumab, cardiotoxicity, sequential or concurrent

1. Introduction: Cardiotoxicity and anthracyclines

Currently, anthracyclines, doxorubicin, and epirubicin remain as the representative key drugs for breast cancer treatment and are the most widely prescribed and effective cytotoxic drugs used in oncology. However, anthracyclines are well known to have severe adverse effects on cardiac function and to cause cardiomyopathy. Congestive heart failure (CHF) induced by anthracyclines depends on the cumulative administered dose and regimen schedule. The mechanism is thought to be direct myocardial injury due to the formation of free radicals and the prevalence of cardiomyopathy increases significantly when patients are given 550 mg/m^2

of doxorubicin [1–3]. In particular, the estimated percentage of patients who develop CHF at a cumulative doxorubicin dose of 400 mg/m^2 is 3%, increasing to 7% at 550 mg/m^2 and to 18% at 700 mg/m^2. It was also reported that CHF is schedule dependent; the incidence was lower with a once-weekly schedule compared with a once tri-weekly schedule. In addition, the prevalence of CHF is increased in the young/elderly, after mediastinal XRT, females, and in those with a history of cardiac disease. Anthracyclines are still used in cancer therapy despite the existence of severe cardiotoxicity because it has irreplaceable anti-cancer effects. In most practitioner guidelines for breast cancer, anthracycline-including regimens are present, and this is also true for the National Comprehensive Cancer Network (NCCN) practitioner guidelines [4]. Due to the cumulative dose limitation and its difficult usage in cancer patients with worsening clinical conditions, we use anthracyclines only for preoperative or adjuvant therapy regimens and avoid using it for recurrent or Stage IV treatments. If we administer the "doxorubicin + cyclophosphamide (AC) cycled once per three-week regimen" according to the NCCN guidelines, doxorubicin is given at 60 mg/m^2 on day 1. Thus, the cumulative dose of doxorubicin would reach 360 mg/m^2 at 6 cycles. If we select the dose-dense regimen in which we administer AC once every 2 weeks, there may be altered cardiovascular risks, and the NCCN guidelines recommend it be stopped at 4 cycles. As described above, we must stop anthracycline regimens before 3 months even if excellent results are observed. Anthracyclines are fated to be administered for preoperative or adjuvant chemotherapy for radical cure, and after the administration of anthracycline-containing adjuvant therapy, they have no role in the treatment of recurrent cancer.

1. Anthracyclines have severe cardiotoxicity, and the prevalence of anthracycline-induced cardiomyopathy depends on the cumulative administered dose and regimen schedule.

2. Cardiotoxicity and trastuzumab

Trastuzumab is one of the most successful chemo-agents for molecular targeting therapy in breast cancer treatment. Amplification of the gene encoding the ErbB2 (Her2/neu) receptor tyrosine kinase and overexpression of Her2/neu protein are seen in approximately 20% of primary invasive breast cancers [5–7]. Trastuzumab (Herceptin®; Chugai Inc., Tokyo, Japan) is a humanized monoclonal antibody with high specificity for the extracellular domain of HER2/neu protein [8]. Single use of trastuzumab demonstrated modest antitumor activity, but in combination with standard chemo-agents (paclitaxel, docetaxel, doxorubicin, cyclophosphamide, and their combinations), trastuzumab exhibits synergic effects for cancer treatment. Indeed, combination protocols containing trastuzumab improved the time to progression, overall response, and duration of response and had a favorable impact on survival in several large-scale clinical trials [9–11].

In patients with metastatic breast cancer, the combination of trastuzumab with standard chemo-agents, anthracycline or taxanes resulted in longer times to progression (TTP), higher response rates, and higher survival rates than with those agents alone. Slamon et al. revealed that the combination of trastuzumab and AC provided the longest median time to disease progression (7.8 months) compared with other three arms— AC alone (6.1 months), paclitaxel

and trastuzumab (6.9 months), and paclitaxel alone (3 months) [12]. However, concurrent administration of AC and trastuzumab also lead to marked cardiotoxicity. The frequency of CHF was the highest in the concurrent AC and trastuzumab arm. The incidence of New York Heart Association class III or IV cardiac dysfunction was 16% in the concurrent AC and trastuzumab arm, whereas that in the AC alone arm was 3%, that in the paclitaxel and trastuzumab arm was 2%, and that in the paclitaxel alone arm was 1%. However, this result was predictable.

In order to administer AC regimens for 7.8 months (approximately 30 weeks), no less than 600 mg/m^2 of doxorubicin as a cumulative dose should be given, but 7–18% of patients will still develop CHF according to a report by Swain in 2001 [1, 3] even if concurrent trastuzumab is not administered. Slamon reported that less than 7% of the patients in the concurrent AC and trastuzumab arm exhibited chemo-induced cardiotoxicity associated with cumulative doses of doxorubicin of up to 550 mg/m^2. In his study, the cumulative dose of anthracycline was not identified as a compensated risk factor. Indeed, 16% as the prevalence of CHF in the concurrent AC + trastuzumab arm was not different from the expected percentage. Careful and regular checkup of cardiac function throughout the therapy, prompt intervention to abort administration if any complications occurred, and subsequent appropriate medical care for cardiac dysfunction likely led to this result.

In 2001, in addition to that by Slamon, one more important report was published. Cook-Bruns surveyed the safety data from 930 patients in trastuzumab-containing clinical trials and surveilled more than 25,000 patients who received trastuzumab in the USA [13]. She revealed that risk factors for cardiotoxicity are mainly related to prior or concomitant anthracycline exposure and that cardiotoxicity is unlikely to be induced by a single use of trastuzumab. Furthermore, she suggested a possible mechanism of cardiotoxicity due to anthracycline and trastuzumab use: anthracyclines directly damage the cardiomyocytes, and the following trastuzumab may interfere with growth and repair after anthracycline-induced damage.

Although HER2 receptor is not expressed in normal human cardiac myocytes, it was found to play an essential role in the developing embryonic heart [14–16]. Histological examination and echocardiography of hearts from adult ErbB2-conditional knockout mice demonstrated ventricular enlargement and increased left ventricle (LV) end-diastolic and end-systolic dimensions (LVEDD and LVESD), consistent with dilated cardiomyopathy [17]. HER2 signaling prevents cardiomyocytes from dilated cardiomyopathy and plays an important role in maintaining cardiac function in the adult heart.

Regardless of the study by Slamon, concurrent use of anthracycline and trastuzumab exhibits synergic anti-cancer effects as compared with concurrent use of paclitaxel. Thus, this regimen has potential, even if it should be limited to no more than 4–6 cycles and be performed with proper and regular monitoring by a physician. These conditions are acceptable for preoperative or adjuvant chemotherapy.

1. Anthracyclines induce cardiomyopathy, and the following trastuzumab may interfere with growth and repair of anthracycline-induced damage. From that perspective, concurrent use of trastuzumab with anthracycline and sequential use of trastuzumab following prior anthracycline have equal risks.

2. In preoperative or adjuvant chemotherapy with proper and regular monitoring of cardiac function, the number of cycles in a regimen can be artificially limited to avoid exceeding the applicable cumulative dose for anthracyclines.

From then on, however, the concurrent use of trastuzumab and anthracyclines has been regarded as a special protocol and is considered by most practitioners to be an "absolute" contraindication.

3. Concurrent use in adjuvant settings

As described above, concurrent administration of anthracycline and trastuzumab in adjuvant settings has the potential to revolutionize the treatment of breast cancer. However, to prevent irreversible cardiac damage, some conditions must be met, including a well-thought-out protocol, not administering anthracycline to its hazardous cumulative dose, and proper and regular monitoring of cardiac performance by specialists.

In 2005, Buzdar designed and implemented concrete strategies to examine this concept [18]. For preoperative chemotherapy, he designed a protocol of concurrent anthracycline (epirubicin) and trastuzumab and demonstrated its excellent efficacy and safety for HER2-positive breast cancer. In this trial, significant pCR rates favoring the trastuzumab plus chemotherapy arm were noted before reaching the planned full sample size, and new recruitment was therefore suspended by their Data Monitoring Committee. Indeed, a marked difference in the two arms was observed in this trial; 26.3% of patients in the chemotherapy alone arm achieved pCR as compared with 65.2% of the patients treated with trastuzumab plus chemotherapy. The cardiac function of enrolled patients was strictly monitored, and repeated evaluation was performed at baseline and after the completion of each regimen. Patients older than 75 or with a history of uncompensated congestive heart failure or a cardiac ejection fraction less than 45% were excluded. As a result, no patients developed drug-induced congestive heart failure.

In our opinion, this spotless outcome of this trial was due to the following:

1. Buzdar started the protocol with weekly paclitaxel + trastuzumab prior to concurrent epirubicin (as FEC75, q3w 75 mg/m^2 Day 1) + trastuzumab in this trial. This order, taxanes followed by anthracycline, was different from protocols by other practitioners at the time.

   In 2003, Bear HD reported the results of the NSABP-B27 trial [19]. He administered four cycles of preoperative AC, which was the standard chemotherapy at the time, followed by adding four cycles of docetaxel. The NSABP-B27 trial demonstrated the efficacy of adding docetaxel regarding pathological response rates and overall survival of patients with operable breast cancer. In addition, two large prospective trials (NSABP B-28 and CALGB 9344) were performed to assess the efficacy of paclitaxel given sequentially after anthracycline-based chemotherapy [20, 21]. At that time, the effects of adding taxanes were unclear, and they were therefore commonly added after AC.

   Why did Buzdar select the uncommon sequence?

In the regimen of AC followed taxanes, AC is administered at the beginning of chemotherapy. In tri-weekly regimens, doses for 3 weeks should be administered at once at the start of chemotherapy. In Buzdar's protocol, the weekly dose of paclitaxel, which has less cardiotoxicity than doxorubicin, was initially administered with the concurrent trastuzumab. Based on data from us and Buzdar, marked downregulation of LVEF due to trastuzumab is likely if paclitaxel + trastuzumab are initially administered [22, 23]. Therefore, the period at the start of paclitaxel + trastuzumab may be used to screen for possible trastuzumab-induced cardiotoxicity. Patients with cardiac dysfunction or cardiomyocyte disorders will be revealed in this paclitaxel + trastuzumab phase. Practitioners must not overlook signs and symptoms during regular monitoring in order to safely proceed to the next anthracycline + trastuzumab phase.

2. Buzdar selected epirubicin instead of doxorubicin, and he administered the low dose of 75 mg/m^2 rather than 100 mg/m^2. As described above, the risk of CHF is highly correlated with the cumulative anthracycline dose. In breast cancer treatment, there are two representative anthracyclines: doxorubicin and epirubicin. At equivalent doses, they both have the same efficacy, but epirubicin has a better cardiac safety profile. Indeed, the doxorubicin-to-epirubicin dose ratio that produces a similar degree of cardiac toxicity is 1:1.8 [24]. The French Adjuvant Study Group (FASG) previously investigated the influence of dose escalation of epirubicin by comparing fluorouracil at 500 mg/m^2, epirubicin at 50 mg/m^2, and cyclophosphamide at 500 mg/m^2 every 21 days for six cycles (FEC 50) with the same regimen except with epirubicin at 100 mg/m^2 (FEC100). After 5 years of follow-up, FEC100 significantly improved the DFS and OS, and the risk of cardiotoxicity by the dose escalation in FEC100 was acceptable [25], even after more than 8 years of follow-up [26]. However, as dose escalation to FEC100 was unconventional at that time, FEC75 was paired with trastuzumab in many anthracycline-containing regimens.

3. This research was performed in preoperative setting; therefore, there should be 1- to 2-month break between the end of FEC75 + trastuzumab and the following 6-month trastuzumab for breast surgery and radiotherapy. Based on Cook-Bruns hypothesis that anthracyclines induce cardiomyopathy and trastuzumab may interfere with growth and repair, this interval should give the damaged cardiomyocytes time to recover. However, it remains unclear whether a 2-month break was sufficient for the cardiomyocytes to return to their former state.

4. Description in the NCCN guidelines about the concurrent use of anthracycline and trastuzumab

Following the disclosure of Buzdar's results, the regimen of weekly paclitaxel with concurrent trastuzumab, followed by FEC75 with concurrent trastuzumab, was described in the NCCN practitioner guidelines as their recommendation for preoperative chemotherapy. However, they also stated that the concurrent use of anthracycline and trastuzumab is a contraindication due to the risk of cardiotoxicity, and thus Buzdar's regimen was treated as an exception. The regimen is in the NCCN guidelines published in 2009 (http://www.nccn.org/professionals/physician_gls/pdf/breast.pdf) [27], and the additional comment is written on the same page:

"Trastuzumab should not be given concurrently with an anthracycline because of cardiotoxicity, except as part of the neoadjuvant trastuzumab with paclitaxel followed by CEF regimen."

This was likely an incentive for Buzdar to establish the cardiac safety of his concurrent regimen to confirm that the concurrent administration improved outcomes, was more precise, and improved the pCR rate in preoperative chemotherapy compared with sequential administration. The American College of Surgeons Oncology Group (ACOSOG) Z1041 trial was carried out, and patients with HER2-positive breast cancer were randomly assigned to the sequential regimen, FEC75 followed by paclitaxel + trastuzumab, or the concurrent regimen, paclitaxel + trastuzumab followed by FEC-75 + trastuzumab [28], and compared. The study results were published in 2013, and marked asymptomatic decreases in the left ventricular ejection fraction (LVEF) during chemotherapy were noted in similar proportions of patients in each group. However, no improvement in pCR rate was observed in the concurrent treatment group compared with the sequential group. Thus, if there is no difference between the performance of concurrent and sequential regimens, concurrent regimens can be avoided. Following the Z1041 trial, the concurrent regimen of anthracyclines and trastuzumab was removed from the NCCN guidelines, and only the statement about contraindication remains.

However, some questions remain unanswered. As a prerequisite for treatment in preoperative and adjuvant settings, sequential delivery of anthracyclines and trastuzumab has not been confirmed as safer than concurrent use. Dang [29] investigated the safety of dose-dense AC followed by paclitaxel + trastuzumab and demonstrated cardiac safety because only one patient (1%) developed CHF and 7% of the patients exhibited asymptomatic LVEF decline during the administration. On the other hand, in the Z1041 trial, LVEF fell below the institutional lower limit of normal for six patients (4–6%) in the concurrent group. The patients in the Z1041 trial did not develop CHF. As a result of this phase II study on 70 patients, the cardiac outcome was not poor, but no better than that for the 142 patients in the concurrent arm in the Z1041 trial. This regimen, dd-AC followed by weekly paclitaxel + trastuzumab, is included in the recommendation by the latest NCCN guidelines for preoperative chemotherapy.

Next, if the initial administration of weekly paclitaxel + trastuzumab can be used to detect potential trastuzumab-induced cardiotoxicity, the dd-AC regimen should be postponed until the weekly paclitaxel + trastuzumab regimen is finished without any cardiac incidents.

In addition, the replacement of the dd-AC regimen with dd-EC regimen or FEC100 regimens is unclear. Concurrent administration of anthracycline and trastuzumab is dangerous, but sequential administration is also dangerous. We should carefully design trastuzumab-containing regimens based on anthracycline dosing, regardless of whether it is concurrent or sequential. The potential risk of trastuzumab-induced cardiotoxicity is not nullified by simply avoiding concurrent use with anthracyclines.

5. Entrance of pertuzumab: TRYPHAENA, NeoSphere, and APHINITY

There has been a recent innovation in anti-HER2 therapy. The new agent "pertuzumab" is a humanized monoclonal antibody that binds HER2 at a different epitope of the HER2

extracellular domain than where trastuzumab binds [30]. This new molecular-targeted agent prevents HER2 from dimerizing with HER3 [31], and trastuzumab and pertuzumab, when given together, exhibit synergic effects to block HER2 signaling, resulting in greater antitumor activity than either agent alone [32]. In the treatment of metastatic breast cancer, the CLEOPATRA trial successfully demonstrated that the combination of pertuzumab + trastuzumab + docetaxel, as compared with the standard docetaxel-trastuzumab regimen, significantly improved progression-free survival, without escalating cardiac toxicity [33, 34].

In the preoperative setting, TRYPHAENA [35, 36] and NeoSphere [37] also nearly doubled the pCR rate, as observed by the synergic effects of trastuzumab and pertuzumab. In July 2017, the APHINITY trial revealed that the addition of pertuzumab to trastuzumab-containing conventional chemotherapy improved invasive disease-free survival as adjuvant treatment [38].

The protocols of these pertuzumab-containing trials were designed before the disclosure of the Z1041 trial; therefore, the patients in the TRYPHAENA trial were given pertuzumab and trastuzumab simultaneously with FEC100 (5-fluorouracil: 500 mg/m^2; epirubicin: 100 mg/m^2; cyclophosphamide: 600 mg/m^2) (**Figure 1**). Of note, the concurrent administration of anthracycline and anti-HER2 agents was performed before the taxane-containing regimen. However, this protocol has a prerequisite cumulative dose of anthracycline (as Epirubicin, 300 mg/m^2; 300 mg/m^2 in the Z1041 protocol), and the patients should have drug holidays after the concurrent administration.

Regarding the efficacy of these protocols, the preoperative pCR rates in the TRYPHAENA trial were 61.6% (ypT0/is) and 50.7% (ypT0/ypN0), and it was 45.8% in the NeoSphere trial. Compared with the concurrent dosing arm in the Z1041 trial (pCR rate was 54.2%), these outcomes were not improved. This is likely because both TRYPHAENA and NeoSphere trials administered the sequential taxane or anthracycline postoperatively, whereas it was given preoperatively in the Z1041 trial. Cardiac safety was maintained in all trials and no patients developed CHF.

The next question is whether the addition of pertuzumab to the Z1041 regimen will escalate the pCR rate in the preoperative setting. If yes, it should surpass the outcomes by TRYPHAENA and NeoSphere. In practice, trastuzumab was given every 3 weeks at 8 mg/kg, followed by 6 mg/kg from its initiation. The pertuzumab loading dose was 840 mg, followed by 420 mg every 3 weeks. Docetaxel was given at 75 mg/m^2 every 3 weeks. After completion of four cycles, eligible patients underwent the next regimen. The following FEC75 therapy (four cycles of fluorouracil at 500 mg/m^2 intravenously, epirubicin at 75 mg/m^2 intravenously, and cyclophosphamide at 500 mg/m^2 intravenously every 3 weeks) was administered with concomitant trastuzumab at 2 mg/kg on days 1, 8, and 15 of the 21-day cycle for four cycles (**Figure 1**). Then, the patients underwent surgery, radiotherapy, and standard hormone treatment for ER-positive patients according to the guidelines. After surgery, and if needed, after radiotherapy, patients should continue trastuzumab for a total duration of 1 year from the start of neoadjuvant therapy.

This regimen, T(rastuzumab)-P(ertuzumab)-D(ocetaxel) followed by FEC75 - T(rastuzumab) has not yet been evaluated in a phase II trial, but its efficacy and feasibility should be predictable as an extension of the previous studies described above. The cardiac feasibility of each regimen, T–P-D and FEC75-T, was established using either regimen alone but was insufficient in combination. However, the anti-cancer performance of this regimen is promising.

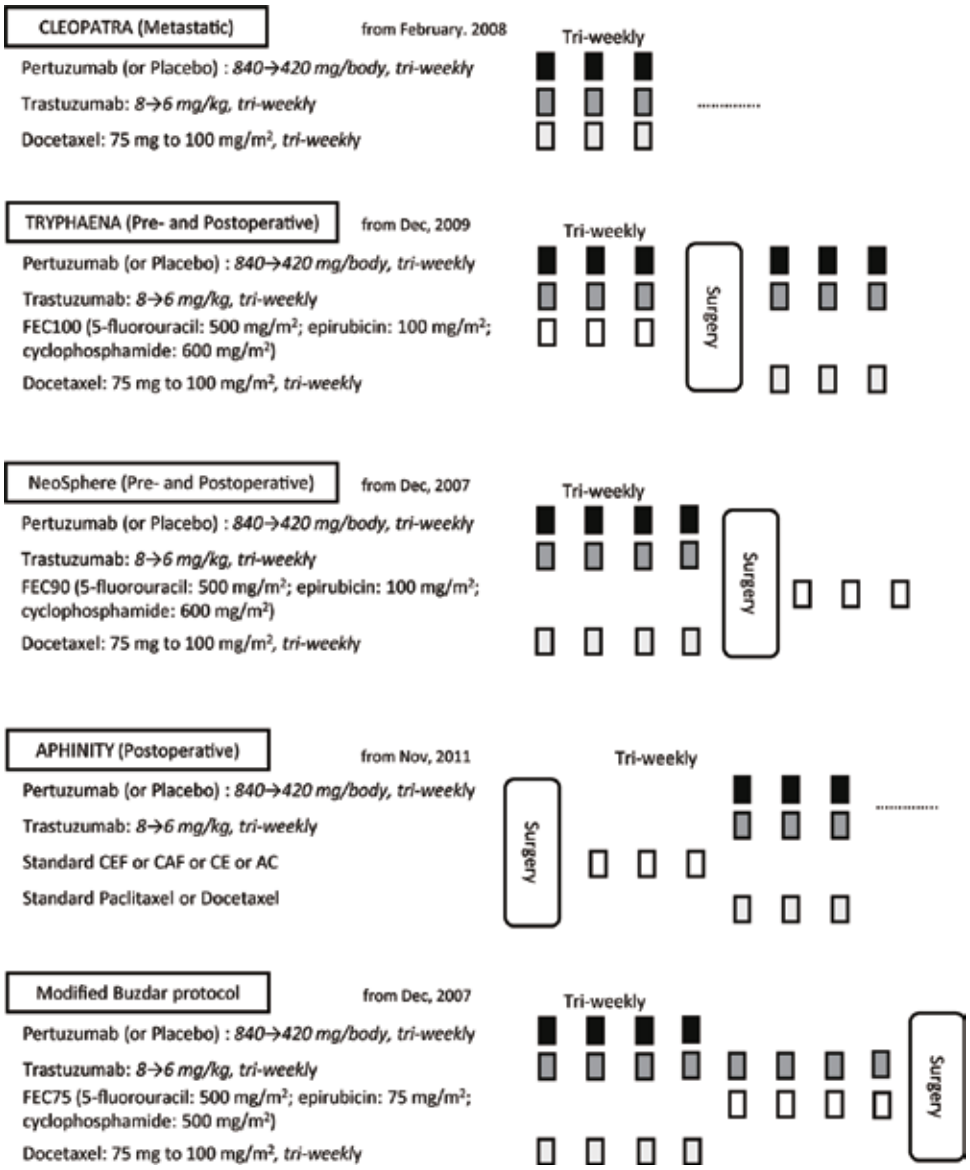

Figure 1. Schema of each protocols, given in this manuscript.

6. Neoadjuvant chemotherapy; Trastuzumab-Pertuzumab- Docetaxel followed by FEC75-Trastuzumab

6.1. Patients and protocol

Following the CLEOPATRA (2010) trial and when the Japanese public insurance began covering pertuzumab (August 2013), we adopted pertuzumab at our hospital and have performed

the T-P-D regimen as the standard chemotherapy for HER2-positive metastatic breast cancer. Prior to this, we performed the Buzdar regimen, weekly paclitaxel + trastuzumab, followed by concurrent FEC75 + trastuzumab, as the standard preoperative chemotherapy for HER2-positive breast cancer. Therefore, since the disclosure of TRYPHAENA (2013) and NeoSphere (2012) trials, we shifted to the following regimen: T-P-D followed by FEC75-T, as the preoperative chemotherapy for patients with advanced breast cancer. After approval from our institutional review board, we have administered this regimen to 24 patients at our hospital between October 2015 and May 2018, and we finished the protocol in 23 (**Table 1**). Except the one recent patient with T1 N0 Stage I cancer, all patients had advanced breast cancer and three had distant metastasis.

The administration of T-P-D was established by the CLEOPATRA trial, and FEC75-T was also established and familiar to us. Thus, we did not design the phase II trial for this protocol, which is why we did not administer pertuzumab concurrently with FEC-T.

Total (n)	24
Age, average ∓ STD (min. – max.)	54.3 ∓ 1.0 (32–70)
Body-mass index, average ∓ STD (min. – max.)	22.2 ∓ 4.1 (17.33–36.68)
Taking drugs for diabetes (+/–)	1/23
Taking drugs for hypertension (+/–)	1/23
Histology	Invasive ductal carcinoma, 23
	Mucinous carcinoma, 1
ER –, PgR – (n)	10
ER +, PgR –	5
ER –, PgR +	0
ER +, PgR +	9
Baseline LVEF (%)[§]	61.7 ∓ 3.2 (55.0–67.5)
Baseline LVDd (mm)	44.8 ∓ 5.9 (27.1–57.6)
Clinical stage I/II/III/IV (total) (n)	1/12/7/3 (23)
Clinical T 1/2/3/4 (total)	1/12/4/6 (23)
Clinical N 0/1/2/3 (total)[Ψ]	9/11/2/1 (23)
Clinical M 0/1 (total)[Ψ]	20/ 3 (23)
Disease free survival (months)[¶]	9.6 ∓ 5.8 (0–22.4)

[§]Left ventricular ejection fraction was measured by echocardiography at primary therapy, completion of each regimen, and after 1 year from the completion of chemotherapy.

[¶]No recurrence of cancer was noted in these 24 patients, and disease-free survival was thus equal to the follow-up period. Three patients had distant metastasis: Case 1 with liver metastasis, Case 2 with bone metastasis, and Case 3 with bone, pulmonary, and mediastinal lymph node metastasis. These metastases have remained at clinical complete response or not progressed.

[Ψ]We routinely performed positron emission tomography (PET) with 18F-fluorodeoxyglucose (FDG) in the patients to access lymph node involvement or distant metastasis at baseline.

Table 1. Characteristics.

All patients underwent an initial cardiac-echogram evaluation, including left ventricular ejection fraction (LVEF) measurements. Patients with a history of congestive heart failure or a cardiac ejection fraction less than 50% were excluded.

Histological confirmation of the invasive tumor was performed on the specimen taken by core needle biopsy or ultrasound-guided vacuum-assisted breast biopsy. The biopsy samples were also examined for HER2 overexpression by fluorescence in situ hybridization (FISH) or 3+ overexpression by immunohistochemistry (IHC) and for estrogen and/or progesterone receptor expression.

Before initiation of therapy, all patients underwent staging evaluation, which included a complete history, physical examination, CBC, chemistry profile, chest radiography, and 18F-fluorodeoxyglucose positron emission tomography (FDG-PET). Tumor size and extension were examined by contrast-enhanced magnetic resonance imaging (MRI-CE). The imaging studies of the tumors were routinely performed at initiation, after T-P-D, and after FEC-T. Tumor shrinkage (objective response) and disease progression were assessed using the RECIST guidelines (version 1.1) [39].

On each hospital visit, we routinely checked the subjective cardiac symptoms and associated objective findings. Cardiac echocardiogram was routinely obtained at baseline, after T-P-D, after FEC-T, and additionally at 12 months after surgery. All echoes were two-dimensional and transthoracic. If abnormal data were obtained, all were interpreted by cardiologists at our hospital. We defined decreased LVEF as an absolute 10-point decrease in LVEF from baseline or an LVEF of 50%.

6.2. Response to chemotherapy

Only 20.8% of patients achieved complete clinical response (cCR) after P-T-D and the rate increased to 54.2% after FEC75-T. Of note, 37.5% of tumors maintained their objective size (stable disease) even after FEC75-T on contrast-enhanced MRI (**Table 2**).

Figure 2 shows the objective responses for each patient throughout preoperative chemotherapy. After the P-T-D regimen, only five patients achieved cCR, but by the addition of FEC75-T, 13 achieved cCR.

Pathological complete response (pCR; Grade 3) was noted in 73.9% of patients, and by adding Grade 2b, the excellent response rate reached 82.6%. Although there were two patients with Grade 0 response, they initially had large tumors (42.7 mm (mucinous carcinoma) and 124.1 mm (solid-tubular carcinoma)).

6.3. Ejection fraction

No incidence of congestive heart failure has been observed, but two patients had an absolute 10-point decrease from the baseline LVEF, one of whom declined in the FEC75-T phase (**Figure 3**). However, all patients maintained EF over 50% throughout chemotherapy, and no patients stopped chemotherapy due to cardiac adverse events. Including the three patients with distant metastasis, we performed surgery on all patients to achieve local control, to

Phase	P-T-D	FEC75-T
Clinical (n = 24) response*		
CR	5 (20.8%)	13 (54.2%)
PR	14 (58.3%)	2 (8.3%)
SD	5 (20.8%)	9 (37.5%)
PD	0	0
Pathological (n = 23) response¶		
Grade 3Ψ		17 (73.9%)
Grade 2 (Grade 2a, n = 2; Grade2b, n = 2)		4 (17.4%)
Grade 1		
Grade 0§		2 (8.7%)

P-T-D: Trastuzumab was given every week at 4 mg/kg followed by at 2 mg/kg from its initiation. The pertuzumab loading dose was 840 mg, followed by 420 mg every 3 weeks. Docetaxel was given at 75 mg/m^2 every 3 weeks. Pertuzumab and docetaxel were administered for four cycles.

FEC75-T: FEC75 therapy (four cycles of fluorouracil at 500 mg/m^2 intravenously, epirubicin at 75 mg/m^2 intravenously, and cyclophosphamide at 500 mg/m^2 intravenously every 3 weeks) was administered with concomitant trastuzumab at 2 mg/kg on days 1, 8, and 15 of the 21-day cycle for four cycles.

We always started our protocol with P-T-D, followed by FEC75-T.*Clinical and pathological response of the tumor was judged according to the RECIST guidelines.

¶Pathological examination was performed after surgery on the surgical specimen.

ΨGrade 3, n = 8; Grade 3 + 3(n,) n = 3; Grade 3 + 3(n) + 3(d), n = 6.

§These two patients had comparably large tumors and their initial sizes were 42.7 mm (mucinous carcinoma) and 124.1 mm (solid-tubular carcinoma), respectively, on MRI-CE. Both patients had lymph node metastasis, but the final pathological examination revealed excellent efficacy for metastasis (Grade 0 + 3(n)).

Table 2. Tumor response to neoadjuvant chemotherapy.

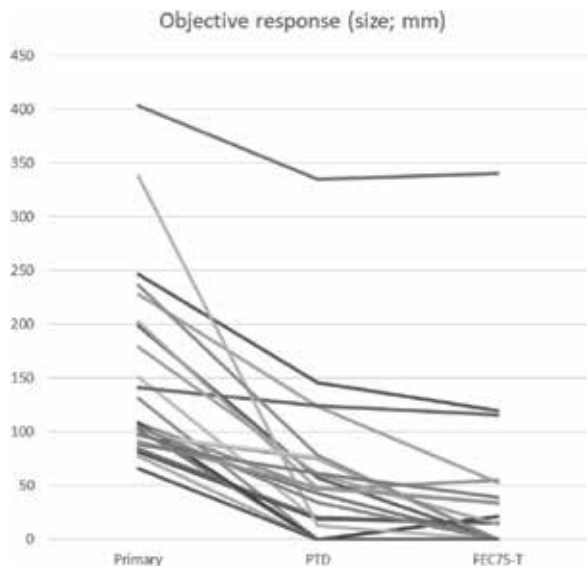

Figure 2. Objective response accessed by contrast-enhanced MRI.

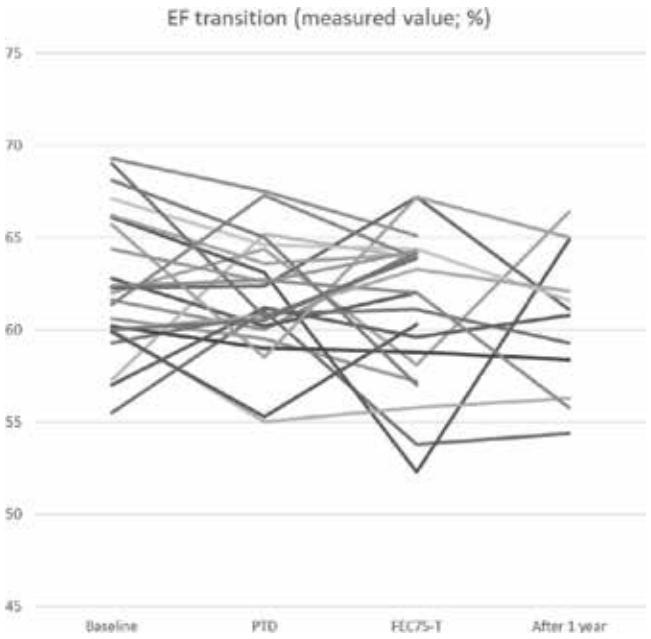

Figure 3. Transition of ejection fraction (EF).

manage discharge, to maintain activities of daily living, and as a surrogate measurement of the response of metastasis.

We have already followed and accessed the EF data of 12 patients for 1 year after the surgery, and no severe adverse events were observed.

6.4. Comprehensive analysis of adverse events

Other frequent complication data are listed in **Table 3**. The patients tolerated the regimen well. We administered pegfilgrastim (recombinant human granulocyte colony-stimulating factor analog filgrastim) to four patients due to febrile neutropenia. The onset of febrile neutropenia occurred in the P-T-D phase and pegfilgrastim was continued until the end of preoperative therapy.

7. Lessons learned from evaluations

Based on our experience, this concurrent regimen: T(rastuzumab)-P(ertuzumab)-D(ocetaxel) followed by FEC75 - T(rastuzumab) may be feasible and powerful, although our sample size was too small to compare with other pertuzumab-containing trials such as TRYPHAENA and NeoSphere. However, as concurrent administration of anthracyclines and trastuzumab is currently contraindicated, the synergic effects of these drugs are unable to be evaluated. Our regimen and its outcome should challenge this current stance.

	Grade 1	Grade 2	Grade 3	Grade 4
Hematological				
Anemia	4 (17.4%)	8 (34.8%)	0	0
Leukocytopenia	2 (8.7%)	5 (21.7%)	6 (26.1%)	0
Neutropenia	2 (8.7%)	3 (13.0%)	5 (21.7%)	3 (13.0%)
Non-hematological, non-cardiac				
Nausea	14 (60.9%)	3 (13.0%)	1 (4.3%)	0
Constipation	8 (34.8%)	2 (8.7%)	0	0
Diarrhea	9 (39.1%)	3 (13.0%)	0	0
Fatigue	16 (69.6%)	3 (13.0%)	0	0
Stomatitis	11 (47.8%)	3 (13.0%)	0	0
Neurosensory disorder	12 (52.2%)	2 (8.7%)	0	0
Cardiac				
Congenital heart failure	0	0	0	0
LVEF measurement				
<10% decrease from baseline, above LLN	12 (52.2%)			
>=10% decrease from baseline, above LLN	2 (8.7%)			
below LLN	0			

LLN = lower limit of institutional normal; 50%.

Table 3. Comprehensive analysis of adverse events.

Buzdar reported in the Z1041 trail that 54.2% of patients in the concurrent group of weekly paclitaxel with trastuzumab followed by FEC75-T had a pathological complete response (n = 142, 45.7–62.6) [28]. In the concurrent group in TRYPHAENA, FEC + T + Pertuzumab→ Paclitaxel + T + pertuzumab, 61.6% had a pathological complete response (n = 72) [35], and in NeoSphere, 45.8% in the sequential group of P-T-D had a pathological complete response (n = 107, 36.1–55.7) [37].

In the concurrent arm in the Z1041 trial, one patient developed Grade 4 cardiac ischemia and Grade 3 left ventricular systolic dysfunction (0.7%), and in TRYPHAENA, symptomatic left ventricular systolic dysfunction (grade ≥ 3, severe adverse event) was noted only in the sequential arm, FEC → Paclitaxel + T + pertuzumab. Although there was no arm for concurrent administration in NeoSphere, one patient in the pertuzumab and trastuzumab arm developed CHF.

Concurrent administration of anthracyclines and trastuzumab can be dangerous, and this was confirmed in the treatment of metastatic carcinoma with indiscriminate continuous dosing. However, sequential dosing is also dangerous. Indeed, it remains unclear whether their synergic toxicity is attributable to their concurrent use and whether we can avoid the cardiac events as long as we use them sequentially. In previous studies on anti-HER2 agents

for preoperative and adjuvant chemotherapy, distinctive characteristic cardiotoxicity was not observed in the concurrent arms.

As described by Cook-Bruns, patients who have received prior anthracyclines are at higher risk for cardiotoxicity even with trastuzumab monotherapy. Prior anthracycline dosing may be the cause of trastuzumab-related heart failure. As trastuzumab and anthracycline are essential agents for HER2-rich breast cancer, we must continue to address this issue from both safety and efficacy aspects. In the TRYPHAENA and NeoSphere trials, detailed monitoring was needed to prevent cardiac adverse events. However, the protocol combining preoperative and postoperative chemotherapy to take drug holidays was complicated for practitioners and patients. The patients undergoing these sequential protocols need an additional 3 months of trastuzumab and hospital visits compared with the concurrent regimen. Moreover, the correlation between the effects of preoperative treatments on pCR and treatment efficacy for survival outcomes becomes unclear.

Contraindication of concurrent use of anthracyclines and trastuzumab has distracted us from its potential efficacy as well as from the inherent danger of anthracyclines together with trastuzumab. Avoidance of concurrent dosing is insufficient. For practitioners, upholding the upper limit of the cumulative dose for anthracyclines, taking every available survey to exclude high-risk candidates, checking and following the patients' cardiac function carefully, and never overlooking the signs of asymptomatic cardiac dysfunction are of the utmost importance.

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Chapter 7

Drug Induced Cardiotoxicity: Mechanism, Prevention and Management

Mina T. Kelleni and Mahrous Abdelbasset

Additional information is available at the end of the chapter

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Abstract

Drug-induced cardiotoxicity is a major adverse effect that has been encountered for some clinically important drugs especially antineoplastic agents. This toxicity has previously led to the post-marketing withdrawal of numerous pharmacologically active drugs and limits the efficacy of other clinically useful ones. Currently, assessing the cardiotoxicity potential is a crucial parameter in drug development, and many models have been established to facilitate its prediction to avoid such toxicity. In this chapter, we will briefly discuss the mechanism of drug-induced cardiotoxicity, risk factors, how to prevent, early detection and/or management from a pharmacological and toxicological point of view.

Keywords: doxorubicin, oxidative stress, mitochondrial dysfunction, risk prediction, biomarkers

1. Introduction

Drug-induced cardiotoxicity is an important cause of attrition of compounds in preclinical and clinical development. It represents one of the most serious side effects associated with novel drug development, and it is known to be one of the major toxic effects induced by several types of drugs [1]. Cardiotoxicity is not restricted to anticancer agents, and almost all therapeutic drug classes have unanticipated cardiotoxicities. However, cardiotoxicity induced by chronically administered drugs, such as neurologic/psychiatric agents and anticancer chemotherapeutic agents, represents a major problem because toxicity may become evident only after long-term accumulation of the drug or its metabolites [2]. Assessing drug-induced cardiotoxicity risk including QT interval prolongation is considered nowadays an integral part of the standard preclinical evaluation of new chemical entities as defined by the

International Conference of Harmonization Expert Working Group for all drugs in development [3]. Strikingly, almost 10% of drugs in the last four decades have been recalled from the clinical market worldwide due to cardiovascular safety concerns, e.g., rofecoxib, tegaserod, and sibutramine, and despite the great efforts to reveal cardiotoxicity in the preclinical phase of development of medicinal products, cardiotoxicity continues to lead safety concerns mainly because of lack of sufficient knowledge of the mechanisms of cardiotoxicity [4].

Currently, cancer is shown to affect more than one in three people in their lifetime, and along with cardiovascular disease, they are the two leading causes of death in developed nations. Thanks to improvement in cancer pharmacotherapy, a current overall 10-year cancer survival stands at 50% across the 20 most common malignancies with a concomitant increase in awareness of the adverse cardiac effects of cancer treatment itself [2]. Further, the 5-year survival rate of US childhood cancers has increased from around 60% in the mid-1970s to more than 80%, and according to the data of National Health and Nutrition Examination, long cancer survivors die, 33% of them, of heart disease [5, 6]. Similarly, cardiovascular mortality has been extensively reported in patients suffering from psychiatric illness, and some antidepressants and antipsychotic drugs have a broad cardiovascular adverse effect profile that can lead to cardiovascular complications, especially cardiac arrhythmias, that have sometimes been shown fatal even to patients with no previous cardiac disease history. For instance, the use of clozapine, the most effective drug for resistant schizophrenia, has been limited due to potentially life-threatening adverse effects, including myocarditis and cardiomyopathy. Clozapine-induced myocarditis has been linked up to 24% mortality. The coexistence of a heart disease complicates the management of mental illness and worsens the illness course, and co-occurrence of psychiatric disorders in cardiac patients might affect the clinical outcome and morbidity [7–9].

Drug-induced cardiotoxicity, commonly in the form of cardiac muscle dysfunction that may progress to heart failure, represents a major adverse effect of some common traditional antineoplastic agents, e.g., anthracyclines, cyclophosphamide, 5 fluorouracil, taxanes, as well as newer agents such as biological monoclonal antibodies, e.g., trastuzumab, bevacizumab, and nivolumab; tyrosine kinase inhibitors, e.g., sunitinib and nilotinib; antiretroviral drugs, e.g., zidovudine; antidiabetics, e.g., rosiglitazone; as well as some illicit drugs such as alcohol, cocaine, methamphetamine, ecstasy, and synthetic cannabinoids. Most of the affected patients had no prior manifestation of the disease [4, 6, 10–12].

Cardiac toxicity of antineoplastic agents includes left ventricular failure, myocardial ischemia, QT prolongation, arrhythmias, pericarditis, myocarditis, hypertension, and thromboembolism. Asymptomatic diastolic dysfunction, which is a common feature observed in many cancer survivors, has been shown to be the earliest noticeable cardiac abnormality [12, 13]. Subclinical cardiotoxicity should also be kept in mind, and it is commonly defined on cardiac imaging as clinically asymptomatic left ventricular systolic dysfunction with a fall in left ventricular ejection fraction by >10% points to a value of less than <50% which is commonly used as the decision threshold to define cardiotoxicity [2]. Alternatively, cardiac dysfunction related to cancer treatment has been also defined as a decrease in left ventricular ejection fraction by ultrasound greater than 10% (from baseline) and with an absolute value

less than 53%, confirmed by a repeat examination at 2–3 weeks. Left ventricular ejection fraction between 53 and 73% is considered normal [14]. Clinically manifested cardiotoxic effects may occur acutely at any time from the first dose of treatment till 2 weeks after it is terminated or chronically that may occur months after termination of treatment [15, 16]. The frequency to recognize such complications with cancer patients has significantly increased during the past few decades due to increased survival of those patients which has accompanied the advances in drug treatment and health care, and early detection of drug-induced cardiotoxicity is of pivotal importance to avoid further deterioration of cardiac function [17].

2. Mechanisms

The exact mechanism of antipsychotic-, anthracycline-, or drug-induced cardiotoxicity remains unclear, though it is likely to be multifactorial [2, 18]. Understanding the mechanisms of anthracycline-induced cardiotoxicity is crucial for effective cardioprotection. Several complex mechanisms have been implicated including formation of iron complexes that can react with O_2 to form $\bullet O_2-$, which in turn dismutates to H_2O_2 and/or enters the Haber-Weiss reaction, resulting in •OH, or can react with H_2O_2 yielding directly •OH; increased production of reactive oxygen and reactive nitrogen species; lipid peroxidation; inflammation; induced cardiomyocyte apoptosis; interstitial fibrosis; abnormal signaling of epidermal growth factor as well as beta-arrestin; inhibition of nuclear topoisomerase II β; induced DNA damage; inhibition of vascular endothelial growth factor signaling; defective mitochondrial biogenesis; and calcium overloading [15, 16, 19–22]. Mitochondria have an essential role in myocardial tissue homeostasis, and deterioration in mitochondrial function eventually leads to cardiomyocyte and endothelial cell death and consequent cardiovascular dysfunction [4]. The antiretroviral nucleoside reverse transcriptase inhibitors, e.g., zidovudine, may cause cardiac mitochondrial dysfunction through inhibition of DNA polymerase-gamma and induction of mitochondrial DNA mutations leading to cardiomyopathy [23]. Two broad categories of cardiac adverse effects are known, functional and structural effects. Noteworthy, seriously altered function may be completely dissociated from structural effect, especially at an early stage [24]. Other than the functional deterioration, anthracyclines were also shown to damage several major structural proteins regulating cardiac muscle contractility including titin, the myofilament forming protein that regulates cardiac function leading to systolic and diastolic dysfunction [12]. In addition, there is a considerable interindividual variability in the susceptibility to chronic anthracycline-induced cardiotoxicity; genetic variants were suggested to have an impact on the occurrence of drug-induced cardiotoxicity; a potential role for polymorphisms in several candidate genes related to the metabolism of anthracyclines, detoxification of free radicals, or variations in body iron levels and genetic testing was recommended to reduce the incidence of anthracycline-induced cardiotoxicity [10, 21, 25, 26]. Anthracyclines are more likely to produce irreversible (type 1 drug-induced cardiotoxicity) microstructural lesions of cardiomyocytes leading to necrosis and apoptosis. Noteworthy, reversible (type 2) cardiotoxicity is associated with biological drugs targeting proteins regulating cancer cell proliferation which are also necessary for maintenance of cardiovascular homeostasis, and this toxicity can

be resolved after completion of therapy or even during its continuation, and overlap as well as addition may occur between the types while using multiple potentially cardiotoxic drugs [16, 22]. Noteworthy, some authors mention that reversible drug-induced cardiotoxic adverse effects are type 1 while irreversible ones are type 2 [2].

3. Risk factors

Patients undergoing chemotherapy have a higher risk of developing cardiovascular complications (**Table 1**), and the risk is even greater if there is a history of heart disease or concomitant radiotherapy, increasing the incidence of events by 30% compared to the general population [5, 27]. The time course of cardiotoxicity varies depending on several factors including patient age at time of exposure and the class effect of chemotherapy drugs, where childhood cancer survivors experience exponentially rising risk for delayed cardiovascular events while adult cardiovascular risk manifests earlier and depends on the number of conventional coexisting cardiac risk factors especially hypertension [2]. Patients who have a moderate to high risk of developing or are suspected to have cardiotoxicities indicated according to their medical history or abnormal imaging and biomarker levels might warrant treatment of risk factors, alternative cancer treatment options, and administration of cardioprotectants [28].

Identification of patients at risk of drug-induced cardiotoxicity is currently considered one of the main objectives for cardiologists and oncologists to personalize cancer therapy and arrange early preventive interventions. It is of key importance to optimize and standardize

Risk factors for drug-induced cardiotoxicity:
Patient age at time of exposure (below 4 years and old age)
Female gender
Black ethnicity
Class of chemotherapeutic agent
Total cumulative dose
Concomitant radiotherapy/cardiac irradiation
Abnormal cardiac imaging or biomarkers levels
History of heart disease or left ventricular dysfunction
Hypertension
Obesity
Diabetes
Dyslipidemia
Physical inactivity
Smoking
Genetic predisposition

Table 1. Risk factors for drug-induced cardiotoxicity.

the management of these patients, in a multidisciplinary approach; an integrated approach using molecular, imaging, and clinical data may allow the selection of patients at risk of developing chemotherapy-related cardiotoxicity. Risk factors also include total cumulative dose, Down syndrome, female gender, black ethnicity, age below 4 years, old age, hypertension, obesity, diabetes, dyslipidemia, physical inactivity, smoking, concomitant cardiac irradiation, concomitant treatments, previous left ventricular dysfunction or cardiovascular comorbidity, and genetic predisposition. However, drug-induced cardiotoxicity may occur idiosyncratically without obvious risk factors [6, 12, 22, 27, 29].

4. Prevention and biomarkers

Measurement of cardio-specific biomarkers can be a valid diagnostic tool for early identification, assessment, and monitoring of cardiotoxicity. Advantages of biomarkers include being minimally invasive, less expensive than echocardiography or nuclear techniques, and can easily be repeated without irradiation of the patient. Moreover, the interpretation of the results does not depend on the expertise of the operator, thus avoiding the possibility of interobserver variability [30].

Moreover, there is an increased interest on the most recent noninvasive diagnostic biomarkers to early predict and follow up anthracycline-induced cardiotoxicity allowing a potential cardioprotective intervention before irreversible damage. Currently, the most important biomarkers are cardiac troponins, brain natriuretic peptide, and N-terminal fragment of brain natriuretic peptide [16, 31]. Persistent elevation of cardiac troponin I concentrations 1 month after anthracycline therapy had more cardiac adverse events at 3 years (84%) than patients with transient or no cardiac troponin I elevations (37 and 1%, respectively). Similarly, cardiac troponin T concentrations are associated with cardiac outcomes in children receiving moderate-dose anthracyclines for high-risk acute lymphocytic leukemia, and elevated concentration of N-terminal pro-brain natriuretic peptide during the first 90 days of therapy was associated with an abnormal left ventricular thickness-dimension ratio 4 years later, suggesting pathologic left ventricular remodeling [6]. Recently, circulating microRNAs are being considered to represent promising noninvasive and specific circulating biomarkers of several cardiovascular diseases, and they were successfully tested in children and young adults treated with anthracycline chemotherapy [32]. Fatty acid-binding protein 3 is a cytosolic protein found primarily in the heart but also in the muscle, brain, and kidney with a primary role in intracellular transport of long-chain fatty acids and in regulation of gene expression via peroxisome proliferator activator receptor. It has been demonstrated to be more sensitive than cardiac troponin for detection of ischemic injury and ongoing cardiac injury associated with congestive heart failure [33, 34]. Other less commonly used biomarkers include plasma cystatin-C, galectin-3, interleukin-6, tumor necrosis factor-alpha, and high-sensitivity C-reactive protein [6]. Noteworthy, the use of the classical biomarkers of lactate dehydrogenase and creatine kinase and their isozymes as biomarkers of cardiotoxicity has been substantially limited due to lack of tissue specificity and sensitivity [35, 36]. However, circulating biochemical markers carry potential problems related to sensitivity and specificity as they can be significantly influenced

by multiple microenvironmental factors and noncardiovascular diseases. Further, troponin levels increase in the blood only after tissue damage has occurred, and thus they cannot be used as early diagnostic markers of dysfunction onset [2, 32]. Human embryonic stem cell-derived cardiomyocytes are under investigation to predict drug-induced cardiotoxicity [3].

5-fluorouracil is perhaps the second, after doxorubicin, the most common cause of chemotherapy-associated cardiotoxicity which occurs predominantly in the first 72 h of the initial treatment cycle and may include chest pain, ECG changes, arrhythmia, pulmonary edema, myocardial infarction, and, rarely, cardiac arrest. Careful clinical monitoring during 5-fluorouracil administration in patients with preexisting cardiac disease is important, and its administration should be stopped immediately in patients who develop a cardiac adverse event. These patients should not be retreated with this agent, as the risk of a subsequent, potentially more serious cardiac event increases with repeat exposure [37].

5. Management

Standard management during anthracycline-based chemotherapy involves cardiac function assessment prior to treatment, monitoring potential cardiotoxicity during the therapy, as well as a long-term follow-up after the chemotherapy is completed [12]. A risk prediction model to identify patients at increased risk for therapy-induced cardiac disease prior to starting or during therapy using patient demographics (e.g., age at treatment, gender), treatment (e.g., cumulative anthracycline dose, radiation exposure), genomics, serial biomarkers, and echocardiographic measurements at baseline and during follow-up is under progress to enable investigators with an interest in cardiac late effects resulting from childhood cancer treatments to perform further investigation in the field [38].

Cardiac damage initially occurs in a molecular phase, followed by cellular damage, asymptomatic dysfunction, and finally symptomatic clinical dysfunction. The diagnostic intervention involves monitoring left ventricular ejection fraction by ultrasound, multigated acquisition scan, or MRI, considering <53% as abnormal. Cardiac MRI is considered the reference technique for quantification of left ventricular ejection fraction. However, ultrasound offers the advantages of its availability, low cost, lack of radiation, and overview of cardiac function. Yet, 2D ultrasound depends on the quality of the image and the expertise of the operator. Furthermore, it has a reported variability of about 10%, similar to the value used for diagnosis of cardiotoxicity [39].

Doppler speckle-tracking-derived longitudinal strain echocardiography has been useful in assessing adults for cardiac damage, and cardiac MRI has characterized myocardial tissue and assessed perfusion abnormalities independent of a good transthoracic window to obtain acceptable echocardiographic images [6]. Radionuclide angiography is an alternative method to detect cardiotoxic damage, and scintigraphy is also used for heart imaging in oncology, as it enables the assessment of left ventricular function [12]. Future improvement in the modalities to early detect drug-induced cardiotoxicity may include advanced techniques in cardiac nuclear molecular imaging and photoacoustic imaging [2].

Some authors have suggested patients receiving cardiotoxic drugs to be regarded as stage A heart failure, and if they experience an asymptomatic decline in left ventricular ejection fraction, they should be treated as per stage B [40]. Several protocols have been proposed to ameliorate or treat doxorubicin-/drug-induced cardiotoxicity including the use of epirubicin which has a less cardiotoxic potential than doxorubicin, reduction of lifetime cumulative dose, avoiding bolus, the use of continuous infusion with reduced peak concentration, prolongation of infusion time, the use of non-pegylated liposomal formulation of doxorubicin to help more specific delivery to the target site which can further reduce adverse cardiac effects, and the concomitant use of the antioxidant and iron chelator dexrazoxane, angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, beta blockers, ranolazine, metformin, and hydroxymethylglutaryl-coenzyme A reductase inhibitors which have been shown to reduce drug-induced cardiotoxicity when used in a prophylactic setting. Further, the use of antioxidants, e.g., self-nanoemulsifying formulation of quercetin, Q10 coenzyme, vitamin E, and carnitine as well as avoidance of combinations which have an augmented cardiotoxic potential like anthracyclines added to trastuzumab would help to reduce the likelihood of drug-induced cardiotoxicity [12, 19, 21, 29, 41–43]. Carvedilol beta and alpha 1 blocker with strong antioxidant properties has been also shown to inhibit anthracycline- and/or trastuzumab-induced left ventricular dysfunction by ameliorating reactive oxygen species formation, mitochondrial alterations, and cardiomyocyte-induced apoptosis. Further, the combination of enalapril and carvedilol seemed to be beneficial in treating anthracycline-induced cardiotoxicity [22]. Additionally, a telemedicine system has allowed interdisciplinary management of the patients receiving anthracyclines with an expert cardiologist, and various chronoprogrammable drug delivery systems have been developed [2, 44]. Noteworthy, dexrazoxane has been reported to augment the myelosuppressive properties of doxorubicin, and a hypothetical concern of its possibility to provide a protective benefit to the neoplastic cells has resulted in its underutilization [45]. Further, it has been recommended that clozapine dose should be titrated gradually (in up to 25 mg/day increments) over 4–6 weeks until the target dose is reached as it has been shown that the risk of clozapine-induced myocarditis may be increased with a higher cumulative dose early in clozapine titration [18].

Interestingly, epidemiological studies have demonstrated that the incidence of cardiovascular disorders in France is strikingly lower than other western countries with a fat-containing diet. This so-called French paradox has been attributed to moderate consumption of red wine containing the potent antioxidant resveratrol in France, and resveratrol was suggested to be used during early cellular damage in organ toxicity after doxorubicin treatment in cancer patients, and its combined use with doxorubicin was suggested to be a viable chemotherapeutic modality that can selectively destroy tumors while concurrently limiting cardiac damages. Resveratrol was shown to mitigate the doxorubicin-induced cardiomyocyte apoptosis, autophagy, and fibrosis [15, 46].

Remote ischemic conditioning, a noninvasive nonpharmacological treatment delivered via a blood pressure cuff as short bursts of ischemia and reperfusion in a peripheral limb, with an unclear mechanism is a potentially cardioprotective treatment that is currently under investigation in cancer patients that may involve a humoral and neural pathway that confers cardioprotection by activating innate pro-survival pathways that ultimately modulate common

mechanisms in ischemia-reperfusion injury and anthracycline cardiotoxicity such as calcium overload, lipid peroxidation, ROS generation, and mitochondrial function [2].

6. Conclusion

Cardiotoxicity is an important dose-limiting side effect of various anticancer agents. In this chapter, we have identified some of the important and commonly used chemotherapeutic and biologic agents that have been reported to be associated with cardiovascular adverse effects. We have discussed the common mechanisms thought to be involved with such toxicity as well as the most important measures used to prevent and manage drug-induced cardiotoxicity. Although cardiotoxicity can occur without any predisposing factors, various risk factors are now known and considered of utmost importance to identify and manage before and during treatment, and we have discussed them. Finally, identifying drug-induced cardiac adverse effects as early as possible will help to prevent irreversible cardiac damage and to ameliorate the long-term morbidity and mortality rates as well as to improve the patients' quality of life.

Conflict of interest

None to be declared.

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Chapter 8

New Biomarkers in Screening Anthracycline-Induced Cardiotoxicity Only with Peripheral Blood Sampling

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Abstract

Because oxidative stress after administration of doxorubicin was identified as playing a central role in cardiac dysfunction, we hypothesized that the expression (or overexpression) of TLR2 and TLR4 contributes to the pathogenesis of doxorubicin-induced cardiac dysfunction. Toll-like receptors (TLRs) are members of the interleukin-1 receptor family (IL1) and are involved in the ability to react to the molecular trigger associated with pathogenic microorganisms. Recent studies have shown that TLR receptors are activated by endogenous signals, such as heat shock proteins and oxidative stress, which can contribute to congestive heart failure. Until recently, the best detection method for cardiotoxicity induced by anthracyclines was myocardial biopsy. Other early screening and early diagnosis methods (biomarkers—cardiac troponins and natriuretic peptide) have not yet proven their efficacy. Our proposed method is a new, revolutionary one that does not imply any kind of physical (and psychic) aggression on the patient: the targeted genetic (TLR2/TLR4) analysis of the human peripheral blood (which is a minimally invasive procedure).

Keywords: anthracycline, cardiotoxicity, doxorubicin, Toll-like receptor, biomarker

1. Introduction

Immunotherapy for cancer has been and still is a subject of intense research since the nineteenth century when Coley noticed that bacterial components can contribute to cancer regression and can cause an immune antitumor immune response.

Permanent cardiotoxic effects of conventional cytostatic therapy underscore the need for early diagnostic methods with great sensitivity to allow early detection of signs of cardiac dysfunction, the consequence of the cardiotoxic effects of cytostatic medication. The specificity of any test should allow for a precise risk/benefit analysis, a balance between the probability of cardiac dysfunction due to high drug doses and the consequences of stopping antitumor therapy.

Several Toll-like receptors (TLRs) are expressed in cardiomyocytes, including TLRs 2 and 4. Through these TLRs, cardiomyocytes respond to endogenous or exogenous signals that can influence pathophysiological responses to dilated cardiomyopathy. Expression or activation of both TLR2 and TLR4 are overregulated in experimental and in vivo (hypertensive and/or clinical heart failure patients) models. Therefore, inhibition of TLR signaling may be of great therapeutic benefit for dilatation cardiomyopathy (CMD), especially in the treatment with doxorubicin.

2. Serum markers: cardiac troponins and natriuretic peptide

For cardiovascular disorders, including doxorubicin-induced heart failure, an increased number of candidate markers were studied to be useful in detecting myocardial injuries. These include cardiac troponins and natriuretic peptides.

However, most of the results have highlighted the fact that some of the patients have detectable troponin, suggesting that this may be considered as a prognostic marker for doxorubicin-induced cardiotoxicity.

Cardiac troponins are complex joints of thin filaments that are involved in the regulation of actin-myosin coupling in the cardiac muscle and consist of three subunits: troponins T, C, and I [1–4].

Cardiac troponins T and I are both highly sensitivity and have specificity for myocardial injuries [5]. Both markers are used as prognostic and diagnostic tools in acute coronary syndrome [6]. Cardiac troponins have also been studied from the perspective of use as markers for anthracycline-induced cardiotoxicity, with bivalent results [7].

These contradictory results can be determined by many factors, such as heterogeneous population studies, variable cumulative doses of anthracycline, and different study protocols with different working methods.

The utility of troponins was mainly demonstrated after administration of antineoplastic and β-sympathomimetic drugs, although the routine use of these markers in monitoring in patients receiving anthracycline therapy is far from being solved.

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3. Toll-like receptor and cardiotoxicity

Toll-like receptors (TLRs) are members of the interleukin-1 receptor family (IL1) and are involved in the ability to react to the molecular trigger associated with pathogenic microorganisms. Recent studies have shown that TLRs are activated by endogenous signals, such as heat shock proteins and oxidative stress, which can contribute to congestive heart failure.

Oxidative stress is one of the major doxorubicin (Dox)-induced cardiac dysfunctions. Thus, we hypothesized that TLRs contribute to the pathogenesis of doxorubicin-induced cardiac dysfunction.

Toll-like receptors (TLRs) recognize pathogens associated with molecular patterns such as lipopolysaccharides, peptidoglycan, bacterial lipoproteins, and oligonucleotides during inflammatory response.

TLRs have interleukin-1 (IL1)-like response pathways as an intracellular signaling means leading to the nuclear localization of the kb/Rel-type nuclear transcription factor (NF). Moreover, TLRs are expressed in various organs, such as the lung, brain, kidneys, heart, etc.

Recent studies have suggested that the "activated myocardium" through mediated TLR signaling pathways in response to exogenous ligands induces myocardial dysfunction.

Other studies analyzed by us for scientific documentation have also demonstrated that there are other signaling-mediated TLR pathways that are activated by endogenous signals such as heat shock protein and oxidative stress in cardiomyocytes isolated from ventricular level.

We must also keep in mind that it has recently been shown that TLR2 plays an important role in ventricular remodeling after myocardial infarction and also with cardiac specificity (TLR4 compared to it does not have a system/organ specificity).

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These studies also indicate that free radicals play an important role in doxorubicin-induced cardiotoxicity.

Because oxidative stress after administration of doxorubicin was identified as playing a central role in cardiac dysfunction, we hypothesized that the expression (or overexpression) of TLR2 and TLR4 contributes to the pathogenesis of doxorubicin-induced cardiac dysfunction.

Successful activation and maturation of specific tumoral immune cells are known to be mediated by bacterial endotoxin, which activates Toll-like receptor 4 (TLR4). TLR4 is expressed

on a variety of immune and tumor cell types, but its activation may have opposite effects. While activation of TLR4 may promote antitumor immunity, it can also cause excessive tumor growth and immunosuppression.

However, TLR4 binding to endotoxin, as well as endogenous ligands, is a notable contribution to the outcome of treatment of various cancers, such as radiation or chemotherapy. Further research into the role and mechanisms of TLR4 activation in cancer may provide new antitumor adjuvants as well as TLR4 inhibitors that could prevent inflammation-induced carcinogenesis or the cardiotoxic effect of anthracyclines in treatment.

The immune system plays an important role not only in defending against microbial infection but also in controlling and monitoring malignant tumors.

Immune cells scan the tissues with the objective of eliminating newly formed malignant cells before transforming into fully formed tumors. Malignant cells develop complicated mechanisms that allow them to inhibit immune cells by secreting specific cytokines that create an immunosuppressive medium [1]. Tumors can even directly kill the tumor-lymphocyte infiltration, which are CD95 sensitive, by expressing CD95L (Fas ligand) [2].

So, inborn immunity is the first line of defense against microbial infection. The innate immune system cells can recognize the pathogen, and the appropriate immune response is triggered by Toll-like receptors (TLRs), undoubtedly the most important sets of immune-derived vertebrate receptors. TLRs recognize different molecules of microbial origin, called associated pathogenic molecular models. TLRs are located on the cell surface (TLR1, 2, 4, 5, 6) or endosomal compartments (TLR3, 7, 8, 9) with the main role to protect the body from infection. After recognizing these ligands, the TLRs dimerize and trigger a cytoplasmic signaling pathway that leads to the activation of several nuclear factors (e.g., NFκB, IRF) responsible for transcription of immune system genes [3].

Signaling of TLR receptors in immune system cells is critical for regulating innate and adaptive immune response, such as for antigen maturation and presentation as well as for CD8 + and T-cell cytotoxicity, all of which are important factors in antitumor immunity [4]. On the other hand, TLR stimulation may also lead to the proliferation of improved T-cell regulatory and suppressor-like functions in tumor development [5–7].

TLR expression is not limited to immune cells, and indeed many tumor cells have been determined by expression of TLRs, signaling by which they can stimulate tumor growth and immune system evasion [8, 9]. On the other hand, TLR signaling in tumor cells has also been demonstrated to reduce the proliferative capacity of tumor cells [10]. We will focus on reports on TLR4 signaling and its involvement in cancer development and progression.

3.1. Toll-like receptor 4 in health and sickness

TLRs are Toll homologs, insect receptors, which are involved in the determination of dorsoventral polarity during embryogenesis, as well as in the immune response against fungal infections [11, 12]. The first to be found in the human race was TLR4. Endotoxin (i.e., lipopolysaccharides), an external membrane component of Gram-negative bacteria, which is composed of a preserved amphipathic lipid and other variable polysaccharides, is also recognized.

The TLR4 activation mechanism is quite complex and (unlike other TLRs) involves several auxiliary proteins (LBP, CD14) and a coreceptor (MD-2) [3] (**Figure 1**). This is actually MD-2 and not TLR4 that recognizes and binds directly to endotoxin [13, 14]. MD-2 is a soluble protein with a large hydrophobic pocket that represents the binding site for the lipid A lipid chains. Lipid A is usually composed of six acyl chains, but only five of them are bound in the hydrophobic pocket of MD-2.

The sixth acyl chain leaves the pocket and interacts with hydrophobic residues on the TLR4. These interactions are crucial for MD-2/TLR4 heterodimerization and are therefore a prerequisite for TLR4 activation, a cascade signaling [15, 16].

3.1.1. Recommendations for the evaluation of cardiac biomarkers: BNP and cTnI

Recommended method: microparticle enzyme immunoassay (MEIA), quantitative determination of troponin I-cTnI in serum or plasma (troponin I cut-off value <0.04 ng/ml), and quantitative determination of BNP in plasma (BNP-100 pg/mL cut-off value).

Sample collection: from each patient, take 2 ml of fresh blood for each sample collected in plastic tubes; subsequently centrifuge and store plasma/serum samples under optimum conditions at −20°C; and the maximum limit of 2 months is not exceeded.

The number of samples taken from each patient should be two, with a certain periodicity: initially (before initiation of treatment), after the first three cycles of doxorubicin-anthracyclines, and 3 months after initiation of treatment or from inclusion in study.

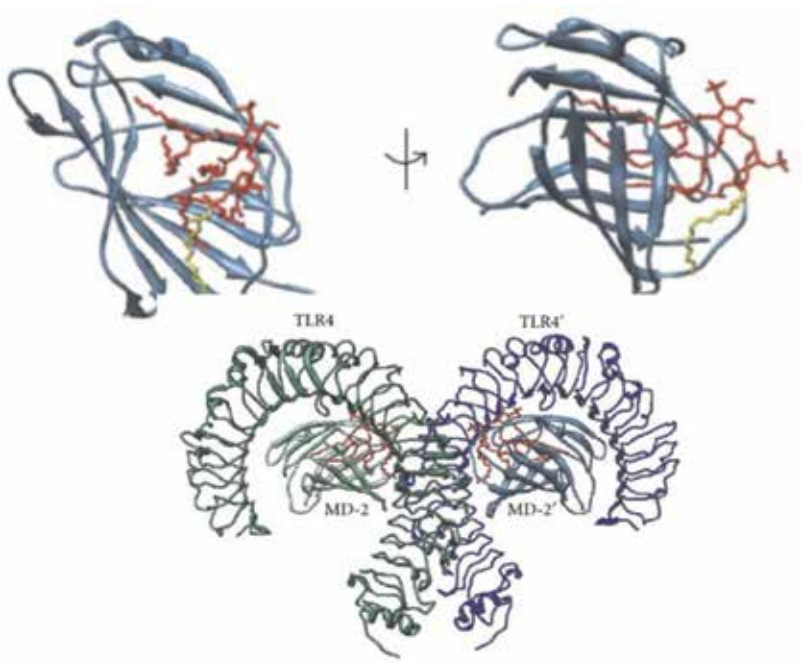

Figure 1. TLR4 spatial representation.

3.1.2. Genetic study for TLR2 and TLR4

As previously mentioned, Toll-like receptors (TLRs) are members of the interleukin-1 receptor family (IL1) and are involved in the ability to react to the molecular trigger associated with pathogenic microorganisms. Recent studies have shown that TLR receptors are activated by endogenous signals, such as heat shock proteins and oxidative stress, which can contribute to congestive heart failure.

Oxidative stress is one of the major doxorubicin (Dox)-induced cardiac dysfunctions. Thus, we hypothesized that TLR receptors contribute to the pathogenesis of doxorubicin-induced cardiac dysfunction.

Toll-like receptors (TLRs) recognize pathogens associated with molecular patterns such as lipopolysaccharides, peptidoglycan, bacterial lipoproteins, and oligonucleotides during inflammatory response.

TLRs have interleukin-1 (IL1)-like response pathways as an intracellular signaling means leading to the nuclear localization of the kb/Rel-type nuclear transcription factor (NF). Moreover, TLRs are expressed in various organs, such as the lung, brain, kidneys, heart, etc.

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Because oxidative stress after administration of doxorubicin was identified as playing a central role in cardiac dysfunction, we hypothesized that the expression (or overexpression) of TLR2 and TLR4 contributes to the pathogenesis of doxorubicin-induced cardiac dysfunction.

3.1.3. TLR2 and TLR4 genetic study methods

For sampling we recommend the use of Tempus ™ Blood RNA Tube tubes (4,342,792, Applied Biosystems®—**Figures 2–4**).

http://dx.doi.org/10.5772/intechopen.79587

Figure 2. Tempus™ blood RNA tubes.

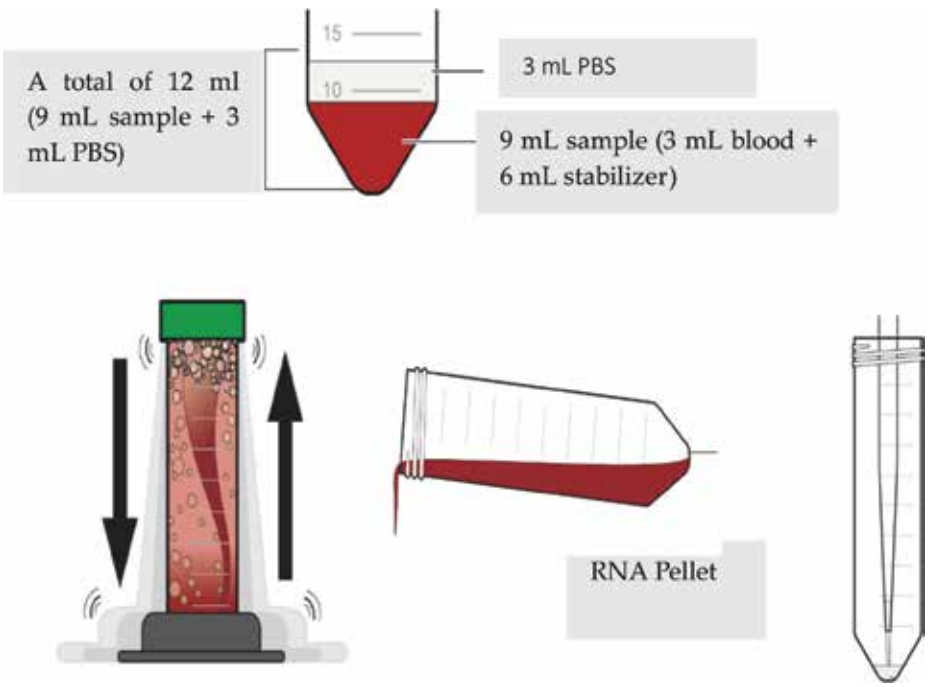

Figure 3. Graphic representation of the RNA isolation procedure.

The tubes contain 6 ml Stabilizing Reagent.

Each tube will harvest 3 ml of peripheral venous blood from patients.

After harvest, the blood is mixed with the cell lysate stabilizing solution at the same time. The stabilizing agent inside the tube contains inhibitors of RNase while maintaining stable gene expression for more than 7 days at 4°C.

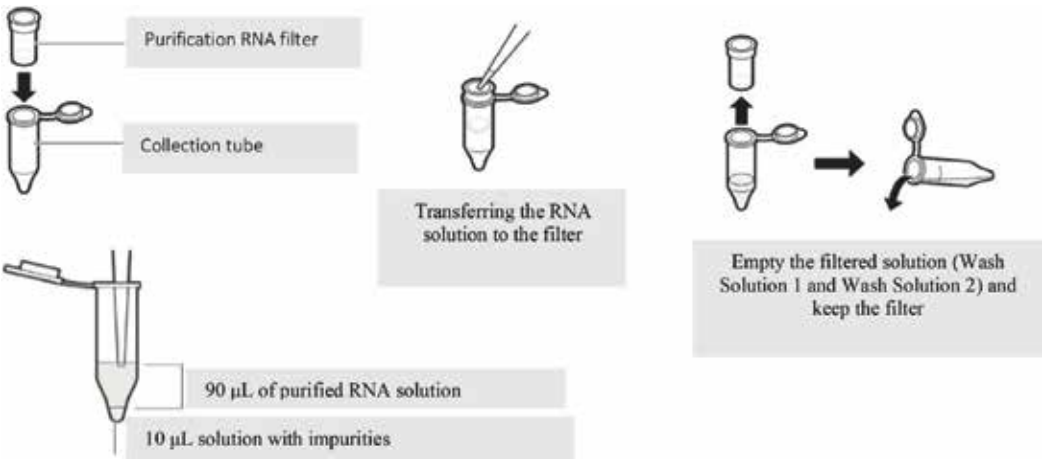

Figure 4. Graphic representation of the RNA isolation procedure.

The stabilizing solution allows the RNA to precipitate by maintaining the DNA and proteins in the solution.

After harvesting, samples should be kept at 4°C until RNA is isolated. Isolation of RNA will take place within 5 days of harvesting.

The RNA isolation procedure includes:

The contents of the Tempus ™ Blood RNA Tube tubes were transferred to 50 mL tubes.

3 mL of PBS will be added.

Stir for 30 seconds after which the samples are vortexed at 3000 g for 30 minutes.

After centrifugation, the supernatant is discarded, and the tube is left in place for 1–2 minutes on an absorbent material.

The RNA pellet is resuspended in 400 µL of Purification Resuspension Solution and vortexed briefly; after resuspension the RNA will be purified.

3.1.4. Obtaining complementary DNA (cDNA)

For the reverse transcription reaction, the High Capacity cDNA Reverse Transcription Kits (4,368,814, Applied Biosystems) kit will be used.

The procedure for obtaining cDNA is:

Two master RT mixes according to **Table 1** will be prepared.

Use 10 µL of 2X RT and 10 µL of RNA for a reaction, to obtain the RNA solution; the RNA samples should be diluted so that in the final 100 ng/5 µL RNA solution 400 ng RNA will be used for a 20 µL reaction volume.

The amplification reaction will be performed using the 2720 Thermal Cycler (Applied Biosystems) according to the specified program (**Table 2**).

Component	Quantity (μL)
10× RT Buffer	2.0
25× dNTP Mix (100 mM)	0.8
10× RT Random Primers	2.0
MultiScribe™ Reverse Transcriptase	1.0
RNase Inhibitor	1.0
Nuclease-free H_2O	3.2
Total volume/reaction	10.0

Table 1. Preparation of 2 × RT master mix with inhibitor/reaction RNase.

	Step 1	Step 2	Step 3	Step 4
Temperature (°C)	25	37	85	4
Time (min)	10	120	5	∞

Table 2. The reverse transcription program.

TLR 2	Sequence	Bp	Tm	GC%	Product length
Forward primer	**GGCATGTGCTGTGCTCTGTT**	20	61.52	55.00	125
Reverse primer	**GCTTTCCTGGGCTTCCTTTT**	20	58.66	50.00	
TLR 4	**Sequence**	Bp	Tm	GC%	Product length
Forward primer	**TTGAGCAGGTCTAGGGTGATTGAAC**	25	62.54	48.00	143
Reverse primer	**ATGCGGACACACACACTTTCAAATA**	25	61.72	40.00	
GAPDH	**Sequence**	Bp	Tm	GC%	Product length
Forward primer	**GCACCGTCAAGGCTGAGAAC**	20	61.57	60.00	138
Reverse primer	**TGGTGAAGACGCCAGTGGA**	19	61.14	57.89	

Table 3. The sequence of used amplicons.

3.1.5. Determination of relative gene expression for TLR2 and TLR4 from isolated RNA samples

For qRT-PCR amplification, the LightCycler 480 SYBR Green I Master kit, which is optimized for the LightCycler 480 Thermocycler, is used. The GAPDH was chosen as the reference gene. The sequence of amplimers used for the experiments is shown in the following table (TLR2 and TLR4 expression in peripheral blood mononuclear cells of patients with chronic cystic echinococcosis and its relationship with IL-10, Parasite Immunology, 2011, 33, 692–696, J.-Y. Shan, W.-Z. JI, H.-T. LI, T. Tuxun, R.-Y. LIN1, & H. Wen) (**Table 3**).

Components	1×
H_2O	3 μl
Primer F	1 μl
Primer R	1 μl
SYBR Green Master	10 μl
Total	15 μl

Table 4. The PCR mix.

Activity	Number of cycles	Temp (°C)	Duration	Fluorescence acquisition
Preincubation	1	96	5 minutes	—
Amplification	45	95	10 seconds	—
		60	10 seconds	—
		72	20 seconds	Single
Melting analysis	1	95	5 seconds	
	1	55	1 minute	
	1	97	—	Continuous
Cooling	1	40	10 seconds	—

Table 5. Staged purification program.

Amplicons should be brought to a concentration of 10 μM and cDNA samples at a concentration of 25 ng/μl.

In a 0.5 mL Eppendorf tube, prepare the PCR mix according to **Table 4**.

From this mix, place 15 μl each well of the multiwell plate, and then add 5 μl cDNA to the appropriate wells. Seal the plate with heat-resistant foil, and centrifuge at 1500 g for 2 minutes. After centrifugation, the plate will be inserted into the LightCycler 480, and the amplification program presented and detailed in this chapter will be used.

After amplification we recommend that the data be analyzed with the LightCycler 480 Software, Basic/Advanced Relative Quantitation (**Table 5**).

4. Explanations and discussion

A simple representation of the concept of a research (or knowledge) system in science and engineering can be imagined as a "black box," considered only in terms of inputs, outputs, and function of the system or process in that system.

The current state of knowledge from the perspective of myocardial immunomodulation during and after (possibly) cardiotoxic treatment with anthracycline (especially doxorubicin) can

be empirically exemplified by this term "black box." I mean, we own a "black box"; we know what goes into this "box"; we know as well what goes out DAR; we do not know what's going on inside this "box."

Thus, we know of the presence of an immunomodulating inflammatory process in response to the anthracycline/doxorubicin molecular response and reaction; we know "cardiotoxic cardiovascular remodeling," but we do not know in detail the mechanisms by which these specific phenomena or specific immunological activators are responsible.

Dilated cardiomyopathy, a common cause of heart failure, is characterized by progressive cardiac remodeling and a decline in cardiac function. Currently, in the literature, the 5-year mortality rate for patients with dilatation cardiomyopathy is 50%. While many studies have documented that dilated cardiomyopathy has both idiopathic and genetic origins, it is being attempted at international academic medical level to demonstrate that inflammation of different origins and manifestations also induces dilated cardiomyopathy. Doxorubicin (Dox) is an effective antitumor agent. Despite its use as a common chemotherapeutic agent, the use of Dox may lead to cardiotoxicity.

Multiple intravenous treatments with doxorubicin over a period of several months have been shown to induce cardiomyopathy in mice and cardiomyopathy in humans [8]. Inborn immune response is the first line of defense and is responsible for the immediate recognition and counteraction of microbial invasion or any agents considered harmful to the body. This component of the immune system consists mainly of phagocytes-macrophages and neutrophils—which ingest and kill pathogens and then transmit information to the adaptive immune system by producing cytokines and chemokinesis by presenting to the lymphocytes the microbial antigen, which leads to the development of a specific response.

This specificity of the adaptive immune response, which is mediated by B and T lymphocytes, is accomplished by somatic mutations and the selection of receptors that most accurately recognize microbial antigens. In contrast, the innate immune response uses "pattern recognition receptors" (PRRs), which recognize highly conserved microbial structures, allowing the host to quickly identify a wide range of pathogens without the somatic mutation time. Generally, each receptor recognizes a series of microorganisms based on ligand specificities. Some receptors also have endogenous ligands and play essential roles in homeostasis (Brown G.D. 2006). Key receptors involved in the recognition of infectious agents and products released by injured or "dying" cells are Toll-like receptors (TLRs).

Activation of innate immune system receptors is followed by rapid changes in gene expression, including genes for cytokines, chemokines, degradative enzymes, and enzymes responsible for the production of small molecule inflammatory mediators. Thus, the released cytokines and chemokines can activate and recruit other cells at the site of the infection or at the site of "the presence of a non-self" (in the case of doxorubicin both by direct effect on receptors and by synthesis and degreasing) finally leading to the activation of the adaptive immune response. In mammals, activation of TLR signaling induces both innate and adaptive immune responses. By phagocytosis of microbial pathogens or non-self agents, TLR membranes (TLR-2, TLR-4) are recruited by phagosomes and are thus activated (mature as self-contained components) through cellular or microbial wall components. This phagosome maturation is also regulated by intracellular signals transmitted by the TLR and ensures the selection of microbial antigens

and their presentation by MHC II molecules. It is imperative that immature innate responses be fine-tuned. Delayed or insufficiently comprehensive responses lead to a failure to control infection or overexpressed inflammatory response in our case (doxorubicin cure). However, excessive or inadequate inflammation may be harmful and even fatal. Hyperinflammatory responses that characterize the body's response to treatment with doxorubicin or other anthracyclines provide a paradigmatic example, such as that excessive inflammation leads to inflammatory bowel disease and arthritis. Endogenous ligands of TLR2 and TLR4 include extracellular matrix products (hyaluronate and heparan sulfate) and molecules released by dead or injured cells (HMGB1, fibronectin, heat shock proteins, fibrinogen, and low density lipoproteins). Many of these molecules accumulate in patients' joints or other inflammatory sites, for example, in the myocardium with subsequent expression through an inflammatory process. The precise mechanism by which the TLR recognizes such a wide range of structures is not yet clarified (Marshak-Rothstein A, 2006). Multiple Toll-like receptors (TLRs) are expressed in cardiomyocytes, including TLR2 and TLR4. By these TLRs, cardiomyocytes respond to endogenous or exogenous signals that may influence pathophysiological responses to induce dilated cardiomyopathy.

Expression or activation of both TLR2 and TLR4 is often expressed in experimental and research models (both human and animal) by hypertension and with proven clinical heart failure [6]. Therefore, inhibition of TLR signaling may be of great therapeutic benefit for doxorubicin-induced cardiotoxic cardiomyopathy. Toll-like receptors (TLRs) recognize pathogens associated with molecular patterns such as lipopolysaccharides, peptidoglycan, bacterial lipoproteins, and oligonucleotides during inflammatory response. TLRs have interleukin-1 (IL1)-like response pathways as an intracellular signaling means leading to the nuclear localization of the kb/Rel-type nuclear transcription factor (NF).

Moreover, TLRs are expressed in various organs, such as the lung, brain, kidneys, heart, etc. Recent studies have suggested that the "activated myocardium" through mediated TLR signaling pathways in response to exogenous ligands induces myocardial dysfunction. Other studies analyzed by us for scientific documentation have also demonstrated that there are other signaling-mediated TLR pathways that are activated by endogenous signals such as heat shock protein and oxidative stress in cardiomyocytes isolated from ventricular level.

5. Conclusions

We must also keep in mind that it has recently been shown that TLR2 plays an important role in ventricular remodeling after myocardial infarction and also with cardiac specificity (TLR4 compared to it does not have a system/organ specificity). Doxorubicin (Dox) is an effective antitumor antibiotic in the anthracycline class. However, doxorubicin also induces cardiomyopathy leading to congestive heart failure, thus often limiting its clinical use.

Doxorubicin-induced cardiomyopathy is mainly caused by increased cardiac oxidant production. It has also been reported that treatment with doxorubicin causes the release of cytochrome C and results in the activation of caspase 3 and cellular apoptosis. These studies also indicate

that free radicals play an important role in doxorubicin-induced cardiotoxicity. Because oxidative stress after administration of doxorubicin has been identified to play a central role in cardiac dysfunction, we hypothesized that the expression (or overexpression exact) of TLR2 and TLR4 contributes to the pathogenesis of doxorubicin-induced cardiac dysfunction.

Conflict of interest

Nothing to declare.

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Cardiotoxicity may be caused by radiotherapy and/or anticancer agents for many malignancies, adverse effects of some drugs in the context of medical intervention or heavy metal intake, especially during the anticancer therapy. This book intends to bring forward the recent development in toxicities from cancer treatment. It updates the possible mechanisms of cardiotoxicities of some anticancer agents and the suggested prevention and treatment strategies. This book contains many valuable contributions from the researchers in oncology and cardiology as well as the clinicians who are experts in this field.

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