

Molecular Mechanisms of Cardiotoxicity: A Review on the Major Side-effects of Doxorubicin

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Doxorubicin is among the most widely used drugs for the treatment of both adult and child cancers. Doxorubicin is the major cause of chemotherapy-induced cardiotoxicity that is dose limiting for the treatment of cancer. Many studies have explored pathophysiology and mechanisms of doxorubicin-induced cardiotoxicity. Cellular and animal experiments proposed that doxorubicin-induced cardiotoxicity mechanism is multifactorial. Oxidative stress has been considered as the primary cause of cardiotoxicity. Although there is no effective treatment for doxorubicin-induced cardiotoxicity currently but many investigations are underway to discover preventive treatments whereas no specific treatment has been approved. Studies have shown that reactive oxygen species and topoisomerase 2b are molecular targets for cardioprotection. Therapeutic imaging methods and cardio-biomarkers may be helpful in the improvement of rapid detection of cardiac damage. In this review, effects of doxorubicin on DNA damage, free radical generation, mitochondrial damage, cell death and other parameters have been studied.

Key words: Doxorubicin, cardiotoxicity, reactive oxygen species, mitochondrial damage, apoptosis

Doxorubicin is a secondary metabolite produced by *Streptomyces peucetius* var. *caesius* and belongs to anthracyclines (ANTs) family. It is an efficient antineoplastic agent used for the treatment of child and adult cancers such as solid tumours, leukaemia, lymphoma and breast cancer. Optimal administration of doxorubicin is hampered due to some toxicity such as hematopoietic suppression, nausea, vomiting, extravasation, alopecia, and cardiotoxicity^[1]. Cytotoxic chemotherapy-induced cardiotoxicity has a high incidence^[2]. Cardiotoxicity included short- and long-term toxic effects in the heart ranging from alterations in myocardial structure and function to severe cardiomyopathy and heart failure that may result in cardiac transplantation or death. Chronic cardiotoxicity occurred after prolonged administration of doxorubicin. Although the possibility of cardiotoxicity development is dose dependent, but it could occur even at lower dose due to individual variations^[3].

Despite frequent attempts, the molecular mechanism of doxorubicin-induced cardiotoxicity has not been identified yet. Although different mechanisms of cardiotoxicity have been described in literature, including DNA damage, alteration of protein synthesis,

formation of oxygen free radicals, cell membrane lesions and lipid peroxidation, mitochondrial damage, release of histamine and catecholamines, induction of immunogenic reactions, calcium homeostasis dysregulation whereas a combination of these factors trigger myocardial lesions^[4,5]. In this report, some of the above-mentioned mechanisms have been reviewed.

Doxorubicin-induced DNA damage:

A major part of the anticancer effect of doxorubicin might be due to irreversible damage of tumour cell DNA. Proposed mechanisms for its antitumor effects included intercalation into DNA that caused prevention of micromolecule synthesis, reactive oxygen species (ROS) generation, DNA binding and cross-linkage and DNA damage by topoisomerase 2b (TOP2b) suppression and induction of apoptosis^[6-8]. TOP2b was recently identified as doxorubicin-induced

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cardiotoxicity mediator in a rat model^[9]. TOP2b unwinds DNA strands during replication, transcription or recombination and is present in all quiescent cells, including cardiomyocytes^[10,11]. Doxorubicin has been known as a TOP2b poison that prevented DNA synthesis by intercalation into DNA strands. TOP2b changes DNA topology, which leads to transient breakage of double-strand DNA and DNA supercoil dysregulation that can result in cardiomyocyte death^[12] (fig. 1).

P53 and apoptotic pathway activation have been shown in doxorubicin-induced cardiotoxicity^[13]. TOP2b is required for P53 activation in response to doxorubicin-induced DNA damage in cardiomyocytes whereas ROS generation from doxorubicin was due to a reduction in the expression of genes of antioxidant enzymes, which were also TOP2b dependent^[14]. Alternatively, once the cell underwent DNA damage, DNA repair pathways get activated. Some of these enzymes cleave oxidized bases before replication, remove oxidized bases from the nucleotidic pool, or remove oxidized bases from DNA after replication^[15,16]. These oxidized adducts were shown to be mutagenic compounds, which lead

to prevention of DNA replication and increased DNA polymerase proofreading error^[17].

Moreover, doxorubicin produced mitochondrial DNA (mtDNA) damage by the formation of adducts with its circular genome that led to mitochondrial machine disruption and mtDNA changes that included rearrangements, deletions and copy number reduction, which were observed in the heart, but not in the skeletal muscle of animal models and patients treated with doxorubicin. This suggested that mtDNA changes might accumulate with time, even in the absence of treatment, which resulted in a deficient respiratory chain that produced more ROS and triggered more mtDNA damage. Although doxorubicin has been toxic to both cancer and normal cells, mechanism of cell death in both cells might not be similar^[18].

Doxorubicin-induced oxidative stress:

Doxorubicin appear to induce oxygen-derived free radical formation through two major pathways: a non-enzymatic pathway that used iron and an enzymatic pathway using mitochondrial respiratory chain^[19,20]. The most commonly accepted theory of doxorubicin-

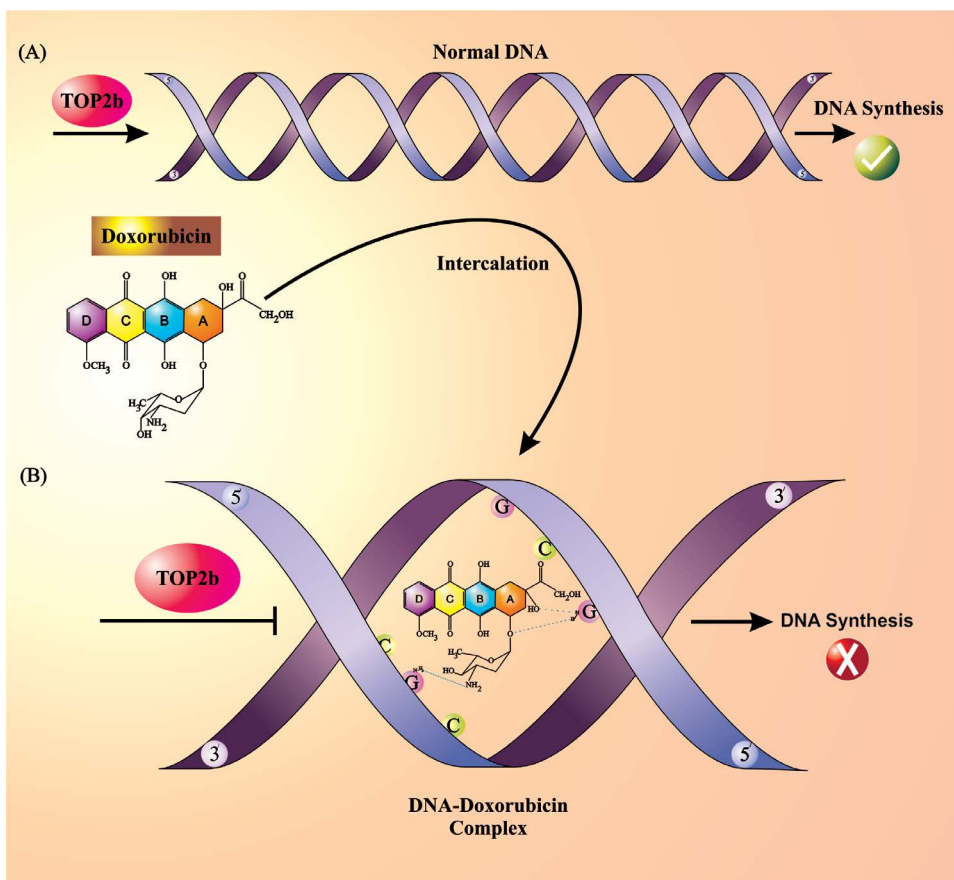


Fig. 1: Doxorubicin intercalation into DNA

A) TOP2b relaxes DNA supercoil to facilitate replication and DNA synthesis, B) doxorubicin forms a complex by DNA through G bases in both of DNA strands and prevents TOP2b activity and DNA synthesis. TOP2b: Topoisomerase 2b, G: guanine, C: cytosine

induced cardiotoxicity implicated the formation of free radicals and superoxides. In the free radical theory, the reaction was initiated by loss of an electron from doxorubicin that triggered the formation of doxorubicin semiquinone radical aided by a reduced flavoenzyme such as NADPH-cytochrome P450 reductase. This radical appeared to be partly stable in the anoxic environment, but under the normoxic condition, its unpaired electron is given to oxygen leading to the formation of superoxide radicals. The semiquinone radical formed a complex with iron that resulted in the free radical complex doxorubicin-iron (Fe^{2+})^[21-24] (fig. 2).

Appropriate flavoproteins such as complex I catalyzes reduced semiquinone radicals by accepting electrons from nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NAD(P)H) and delivering them to doxorubicin. This sequence of reactions was known as the redox cycling, could be very deleterious since a low amount of doxorubicin

has been found adequate for the formation of many superoxide radicals^[25] (fig. 2). This radical damage triggered production of highly toxic aldehydes such as malondialdehyde (MAD). These aldehydes could diffuse easily into the cell through the cell membrane and get attached to micromolecular targets, thus acted as secondary cytotoxic messengers^[26].

NAD(P)H is a large polypeptide complex and researchers have suggested that the presence of definite single nucleotide polymorphism (SNP) in each subunit might make NAD(P)H complex susceptible to doxorubicin. Scientists were successful to show an association of doxorubicin-induced cardiotoxicity with NAD(P)H complex SNPs in non-Hodgkin lymphoma patients^[27]. Doxorubicin-induced chronic cardiotoxicity was associated with an SNP in NCF4 subunit of NAD(P)H oxidase that was found responsible for NAD(P)H complex down-regulation, whereas acute cardiotoxicity was associated with SNPs in P22phox and Rac2 subunits^[27]. Thus, these genetic

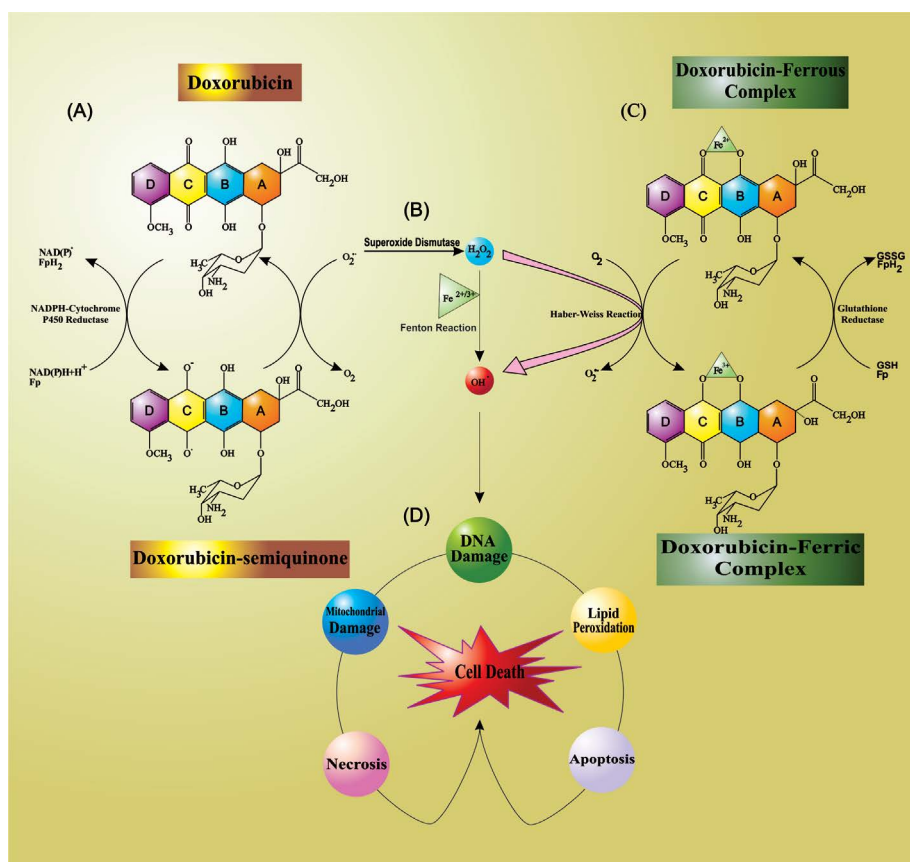


Fig. 2: Doxorubicin-induced oxidative stress

A) Doxorubicin is converted to doxorubicin-semiquinone form by NAD(H)-cytochrome P450 reductase in the presence of flavoproteins, B) generated superoxide is converted to O_2 by semiquinone or to H_2O_2 by superoxide dismutase. H_2O_2 is then converted to OH radical by Fenton and Haber-Weiss reactions, C) the doxorubicin-ferrous complex is converted to doxorubicin-ferric complex by glutathione reductase enzyme in the presence of flavoproteins, D) generated OH radical triggers DNA and mitochondrial damage, lipid peroxidation, necrosis, apoptosis and leads to cell death finally. NAD(P)(H): nicotinamide adenine dinucleotide phosphate, H_2O_2 : hydrogen peroxide, OH: hydroxide, O_2 : oxygen

polymorphisms in NAD(P)H oxidase might serve as a screening tool to diagnose patients with high risk for doxorubicin-induced cardiotoxicity in the future but polymorphisms of other genes could also be important. Some researchers have revealed that doxorubicin-induced cardiotoxicity was associated with CBR3 gene variant V22M in carbonyl reductase domain, a doxorubicin metabolizing enzyme^[28].

Compared to cardiac mitochondria, liver mitochondria lacked the NADH-associated pathway to have an equal reduction from cytosol to the respiratory chain. Therefore, liver mitochondria would not produce enough amount of doxorubicin semiquinone^[29]. Doxorubicin entered the mitochondria and suppressed respiratory chain by binding to cardiolipin, a cardiac specific and polyunsaturated fatty acid-rich phospholipid found in the mitochondrial internal membrane. Cardiolipin has been found to have high affinity to doxorubicin^[30]. Besides ROS, reactive nitrogen species (RNS) were also implicated in doxorubicin-induced cardiotoxicity and there appeared to be a crosstalk between doxorubicin and NO production. Studies have revealed that doxorubicin bound to reductase domain of endothelial nitric oxide synthase (eNOS, NOS3) led to increased superoxide and reduction of nitric oxide formation. Formation of peroxynitrite might also play a significant role in cardiotoxicity^[31]. Continuous administration of doxorubicin *in vivo* has been shown to induce NO production inside of cardiomyocyte with inducible nitric oxide synthase (iNOS, NOS2) over-expression in both mRNA and protein level. In contrast, doxorubicin administration did not change the expression of other two isoforms of this enzyme. Although investigations have demonstrated the involvement of eNOS isoform in doxorubicin-induced acute cardiotoxicity^[32].

Doxorubicin also reduced the activity of cardiac enzymes superoxide dismutase (SOD), glutathione-S-transferase (GST), and catalase (CAT). Upregulation of manganese superoxide dismutase (MnSOD) has been shown to increase cell survival in the presence of doxorubicin as a free radical scavenger in mitochondria^[43]. Calceolarioside protected against doxorubicin-induced apoptosis by upregulating many SOD, heme oxygenase (HO) and potential maintenance of mitochondrial membrane^[43]. Furthermore, it attenuated reduced glutathione level significantly. Generally, antioxidant storage was low in heart tissue compared to other organs in the body that made the heart more susceptible to damage by doxorubicin-induced free radicals^[33].

Role of mitochondria in doxorubicin-induced cardiotoxicity:

The enzymatic pathway for the formation of free radicals was mediated by mitochondria. Doxorubicin has a high affinity for cardiolipin, an abundant phospholipid in the internal membrane of mitochondria. This affinity permitted doxorubicin to penetrate into the cardiomyocytes^[34]. The specific mechanism has not been identified, but the doxorubicin-induced damage to mitochondria likely triggered respiratory chain defect at first that permitted continuous production of free radicals and mitochondrial damage, which might have led to release of cytochrome C triggered apoptosis. P53 pathway activation led to protein translocation into the nucleus and induced changes in gene expression of the proteins to prevent cell division leading in apoptosis^[35]. Doxorubicin triggers activation of various molecular signals from AMP-activated protein kinase that induces apoptosis to affect Bcl-2/Bax apoptosis pathway. By changing the Bcl-2/Bax ratio, downstream activation of different caspases can trigger apoptosis^[36] (fig. 3).

Electron transfer chain proteins are required to bind to cardiolipin for complete function and since doxorubicin disrupted protein-cardiolipin bond, therefore, generated more superoxide (O_2^-). Other mitochondrial membrane proteins such as those are responsible for carnitine transport may be affected by doxorubicin that results in attenuation of mitochondrial function^[37]. These events likely disrupt mitochondrial and cellular metabolism eventually because they produced more than 90% of ATP that is utilised by cardiomyocytes^[38]. This functional damage triggered pathological alterations in the ultrastructure, such as mitochondrial swelling and formation of myelin-like fingers inside mitochondria^[39] (fig. 3).

Other researchers have proposed that targeting of the myocardial energy network to be a part of doxorubicin-induced cardiotoxicity because a significant reduction in phosphate energy pool occurred in cardiomyocytes^[40]. Doxorubicin also reduced respiratory chain complex activity and attenuated function of adenine nucleotide translocator (ANT) or voltage-dependent anion channel (VDAC) or both (responsible proteins for the formation and transportation of ATP from mitochondria to cytosol). Researchers showed that treatment with doxorubicin affected mitochondrial gene expression^[41]. It is likely that doxorubicin-induced alterations might affect gene expression of metabolic enzymes, including oxidative and glycolytic enzymes. They

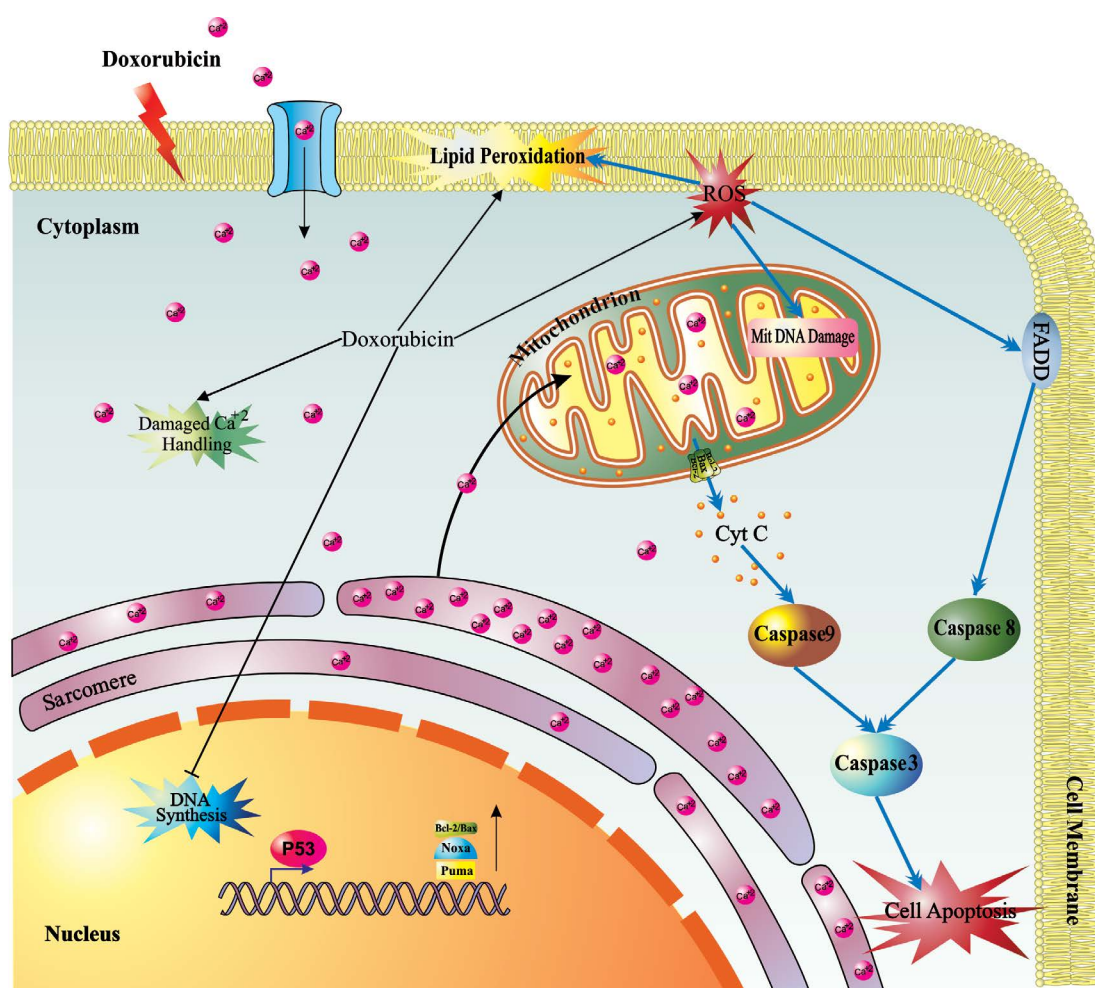


Fig. 3: Doxorubicin effects on cell death

Doxorubicin leads to ROS formation, lipid peroxidation, DNA and mitochondrial damage, impaired calcium handling, induction of P53 and apoptotic pathways. Calcium channel permeability is increased after doxorubicin entry and causes to increase calcium level in sarcomere and cytoplasmic and mitochondrial calcium concentration is then increased and leads to cellular swelling. Upon activation of the P53 pathway, expression of pro-apoptotic proteins (Bcl-2/Bax, Puma, Noxa) is increased that trigger Cyt C efflux from mitochondria to the cytoplasm. Cyt C triggers activation of caspase 9 and then caspase 3 and induces apoptosis. Besides, generated ROS triggers activation of caspase 8 and then caspase 3 and induces apoptosis through interaction with FADD. Furthermore, Doxorubicin blocks DNA synthesis by suppression of TOP2b via intercalation into DNA. ROS: Reactive Oxygen Species, Cyt C: Cytochrome C, FADD: Fas-Associated protein with Death Domain

used transgenic mitochondrial reporter mouse to show that doxorubicin suppressed cardiac mitochondrial metabolism and biogenesis and triggered apoptosis. The mouse was permitted to inhale carbon monoxide (CO) to upregulate the required genes for mitochondrial biosynthesis and nuclear-encoded HO. This revealed that doxorubicin interfered with transcription regulation in both nucleus and mitochondria^[42,43]. Loss of cardiomyocyte following activation of apoptotic and necrotic pathways were an interesting description for doxorubicin-induced cardiotoxicity^[44-46] (fig. 3).

Animal studies have revealed that apoptotic cell death occurred *in vivo* after exposing to doxorubicin. Cell culture studies have also demonstrated doxorubicin-

induced apoptotic and necrotic cell death^[46] (fig. 3). Mitochondrial damage and apoptosis evidence have been found in endomyocardial biopsies of the patients treated with doxorubicin^[47,48]. Biochemical pathways of apoptosis and necrosis have been present in cardiomyocytic death following doxorubicin administration. In doxorubicin-induced apoptosis in the heart, a mitochondrial pathway might have been involved that required Bax, cytochrome C and caspase-3^[49] (fig. 3).

Generally, treatment with doxorubicin increased mitochondrial oxidative stress and disrupted intracellular calcium levels^[50]. Intracellular calcium was raised leading to increased mitochondrial calcium

levels finally^[51]. This increased calcium level led to increase in the permeability of mitochondrial membrane that triggered transmembrane potential disruption, mitochondrial swelling and increased the permeability of its outer membrane to apoptotic factors such as cytochrome C (fig. 3). Caspase activity could be affected by doxorubicin. In the cytosol, cytochrome C formed a complex with adaptor protein apoptosis protease activating factor-1 (APAF-1), dATP and caspase-9, the apoptosome. It has been demonstrated that caspase-3 activation was associated with apoptosis induced by *in vivo* and *in vitro* administration of doxorubicin^[52].

The current hypothesis of necrosis and some forms of apoptotic cell death included the prolonged opening of a conductive pore in mitochondria, the mitochondrial permeability transition pore (mPTP)^[53]. Formation of mPTP has been proposed through conformational changes associated with the binding site of ANT with VDAC between inner and outer membranes of mitochondria^[54]. Recent studies revealed that genetic deletion of ANT and VDAC isoforms did not lead to loss of mPTP, suggesting that none of these proteins were essential components^[55]. In contrast, deletion of cyclophilin D reduced ischemia-reperfusion-induced cell death, suggesting a role for cyclophilin D in mPTP^[56].

The evidence demonstrated that phosphate transporter was an essential component of mPTP and phosphate transporter interaction with ANT regulated mPTP opening. For a long time, it was speculated that mPTP had a role in apoptotic cell death as the protagonist of mitochondrial permeability. Recent data suggested that increased permeability in the mitochondrial membrane has been one of the major factors in necrotic and apoptotic cell death. These data were important since necrosis occurred in many lesions of adult hearts, including cardiotoxic effects of anticancer drugs. In fact, recent experiments have revealed that mPTP did not start apoptosis but played a significant role in necrosis, especially in the heart. Other studies on cyclophilin D deficient mouse have shown that mPTP played an important role in cell necrosis^[57,58].

Overall, these data revealed that opening of mPTP involved in cardiac cell necrosis compared to cytochrome C released in apoptosis. It is well understood that mitochondria played a significant role in the pathogenesis of doxorubicin-induced cardiotoxicity. Prevention of mitochondrial disruption would prevent

myocardial alterations and result in better function of the heart. In addition, more experiments are required to understand the specific function of mitochondria in this pathogenesis.

Doxorubicin-induced cardiotoxicity correlation with apoptosis, necrosis and autophagy:

It has been accepted that doxorubicin-induced oxidative stress activated apoptotic signalling leading to cardiomyocyte apoptosis and both intrinsic and extrinsic apoptotic pathways were involved^[59,60]. It appeared that doxorubicin could induce apoptosis through a mechanism that would not include ROS formation and oxidative stress directly, although apoptosis generated free radicals by itself^[61]. In a doxorubicin model, oxidative stress activated heat-shock factor 1 (HSF1) that produced more heat shock protein 25 (HSP25), which stabilized P53 and increased production of pro-apoptotic proteins (fig. 3).

Heat-shock protein family played a significant role in these processes. These proteins were acted as molecular chaperones that stabilized their target proteins involved in antiapoptotic signalling and prevented cardiac dephosphorylation, ubiquitination, and degradation^[62]. Scientists showed that HSP27 overexpression that had a cardiac protective role against ischemia/reperfusion damage also prevented apoptosis and doxorubicin-induced myocardial dysfunction due to the protective role of HSP27 in the regulation of oxidative stress response and maintenance of mitochondrial function^[63]. HSP10 and HSP60 overexpression also led to an increase in post-translational modification of Bcl-2 proteins that amplify antiapoptotic signalling likely through their effects as molecular chaperones. HSP27 increased AKT phosphorylation (one of the major survival pathways)^[64].

Moreover, some of the HSP are found to be secreted to extracellular matrix, which entered the blood stream and acted as a ligand for toll-like receptors (TLRs). It has been demonstrated that HSP60 signalling could be blocked by antibodies as well as by TLR-2 antagonists against these peptides, whereas HSP70 interacted with TLR-4^[65]. Thus, TLR-2 and TLR-4 roles in doxorubicin-induced cardiotoxicity have been identified. TLR-2 mediated signalling through pro-inflammatory nuclear factor- κ B (NF- κ B) pathway is involved in cytokine production, apoptosis, and cardiac dysfunction after treatment with doxorubicin and deletion of this receptor in knockout mouse maintained

cardiac function and prevented apoptosis. TLR-4 also acted via NF- κ B pathway and knockout mouse showed better cardiac function after treatment with doxorubicin than wild types^[66]. This pathway is not only in cardiomyocyte although it is thought to be specific in the most of the cells. NF- κ B activation by doxorubicin has been demonstrated in endothelial and kidney cells. Furthermore, NF- κ B signalling is not specific for TLRs and initiates regulation of transcription and translation route of many other proteins, including inflammatory cytokines that are likely involved in apoptosis^[67].

Utilization of an NO donor S-nitrosyl-N-acetylpenicillamine (SNAP) produced an antiapoptotic effect by caspase activity suppression through S-nitrosylation in treated cardiomyocytes with doxorubicin. Blocking of volume-sensitive chloride channel has been shown that prevents caspase-3 dependent apoptosis in doxorubicin toxicity^[68].

Cellular necrosis in cardiomyocytes typically has been associated with mitochondrial and cytoplasm swelling, coagulated sarcomer and plasma membrane rupture (fig. 3). This type of cell death could be well controlled by mPTP-dependent mechanisms. ROS rise led to increased mitochondrial calcium level and promoted mPTP opening that caused mitochondrial swelling and reduction of used ATP and thus necrotic cell death is induced^[69].

Autophagy has been considered as a process, which was global degradation and recycling of cytoplasmic components such as aged proteins and organelles^[70]. Autophagy is important in the heart due to turnover of organelles in basal low levels on their normal conditions and upregulation in response to stress, such as ischemia-reperfusion and in cardiovascular diseases such as heart failure. Recent studies have proposed that autophagic cell death might play an important role in doxorubicin-induced myocardial dysfunction^[71]. Overall, mitochondria can be considered as a junction for apoptosis, necrosis, and autophagy processes.

Doxorubicin is an effective chemotherapeutic agent that increased survival of cancer patients, but its administration is hampered due to cardiotoxicity. Cardiotoxicity, an apparent side effect of doxorubicin, might emerge twenty years after treatment as an acute or chronic side effect. This side effect is especially significant for children that have been treated with doxorubicin. Since this side effect is dose restricting and can lead to increased disease severity or even death. Understanding of the mechanisms of action is critical.

It is thought that doxorubicin-induced cardiotoxicity was mediated by oxidative stress.

Until now, the most accepted hypothesis was the free radical theory. Since treatment of cardiac diseases is very expensive, physicians should consider the best cardioprotective option while treating patients with doxorubicin. Administration of cardioprotective agents in an effective option, but development of these effective strategies requires a sound understanding of the molecular mechanisms of doxorubicin-induced cardiotoxicity. Dexrazoxane reduced prolonged cardiotoxicity in cancer patients without leading to secondary malignancies^[72]. Multiple factors interfered with doxorubicin-induced cardiotoxicity including gender, age, drug dose and radiation. Multiple techniques can facilitate monitoring of doxorubicin-induced cardiotoxicity, such as echocardiography, cardiac magnetic resonance imaging and serum biomarkers and all these have been found to be important in the survival of cancer patients.

Many have studied the association of cardiotoxicity with doxorubicin-related metabolism gene polymorphisms. Jensen *et al.* demonstrated that cardiotoxicity could occur in association with demographic characteristics, suggesting a correlation between individual genetics and toxicity development and that many gene polymorphisms are associated with increased cardiotoxicity^[73]. Furthermore, Aminken *et al.* observed that RARG gene variant in the coding region increased the susceptibility of anthracycline-induced cardiotoxicity in children affected with cancer^[74]. Reichwagen *et al.* found that NADPH oxidase gene polymorphisms were associated with anthracycline-induced cardiotoxicity in patients affected with B-cell lymphoma^[75]. Krajnovic *et al.* revealed that ABCC5 and NOS3 gene polymorphisms have been associated with the development of doxorubicin-induced cardiotoxicity in children with acute lymphoblastic leukemia^[76].

Recent data demonstrated that the primary damage of DNA induced by doxorubicin was different from oxidative stress-induced DNA damage and this might be a novel therapeutic target to reduce cardiotoxicity for better therapeutic outcomes in cancer patients. Currently, there is no standard guideline for the treatment of doxorubicin-induced cardiotoxicity. Thus, early detection of doxorubicin-induced cardiotoxicity is essential to prevent subsequent damage. Comprehensive studies are required to investigate biologic parameters,

genetic variations and efficient imaging techniques for prognosis, prevention, and treatment of doxorubicin-induced cardiotoxicity.

Conflict of interest:

The authors report no declarations of interest.

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