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Review article

Molecular mechanism involved in cyclophosphamide-induced cardiotoxicity: Old drug with a new vision

Ashif Iqubal[a], Mohammad Kashif Iqubal[b], Sumit Sharma[a], Mohd. Asif Ansari[a], Abul Kalam Najmi[a], Syed Mansoor Ali[c], Javed Ali[b], Syed Ehtaishamul Haque[a,*]

[a] Department of Pharmacology, School of Pharmaceutical Education and Research, Jamia Hamdard, New Delhi 110062, India
[b] Department of Pharmaceutics, School of Pharmaceutical Education and Research, Jamia Hamdard, New Delhi 110062, India
[c] Department of Biosciences, Jamia Millia Islamia,110025 New Delhi, India

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ABSTRACT

Cyclophosphamide (CP) is an important anticancer drug which belongs to the class of alkylating agent. Cyclophosphamide is mostly used in bone marrow transplantation, rheumatoid arthritis, lupus erythematosus, multiple sclerosis, neuroblastoma and other types of cancer. Dose-related cardiotoxicity is a limiting factor for its use. CP-induced cardiotoxicity ranges from 7 to 28% and mortality ranges from 11 to 43% at the therapeutic dose of 170–180 mg/kg, i.v. CP undergoes hepatic metabolism that results in the production of aldophosphamide. Aldophosphamide decomposes into phosphoramide mustard & acrolein. Phosphoramide is an active neoplastic agent, and acrolein is a toxic metabolite which acts on the myocardium and endothelial cells. This is the first review article that talks about cyclophosphamide-induced cardiotoxicity and the different signaling pathways involved in its pathogenicity. Based on the available literature, CP is accountable for cardiomyocytes energy pool alteration by affecting the heart fatty acid binding proteins (H-FABP). CP has been found associated with cardiomyocytes apoptosis, inflammation, endothelial dysfunction, calcium dysregulation, endoplasmic reticulum damage, and mitochondrial damage. Molecular mechanism of cardiotoxicity has been discussed in detail through crosstalk of Nrf2/ARE, Akt/GSK-3β/NFAT/calcineurin, p53/p38MAPK, NF-kB/TLR-4, and Phospholamban/SERCA-2a signaling pathway. Based on the available literature we support the fact that metabolites of CP are responsible for cardiotoxicity due to depletion of antioxidants/ATP level, altered contractility, damaged endothelium and enhanced pro-inflammatory/pro-apoptotic activities resulting into cardiomyopathy, myocardial infarction, and heart failure. Dose adjustment, elimination/excretion of acrolein and maintenance of endogenous antioxidant pool could be the therapeutic approach to mitigate the toxicities.

1. Introduction

Cancer is a global menace that accounts for 7.6 million (13%) deaths worldwide [1]. By the end of 2017, 1,688,780 new cases of cancer were diagnosed and 600,920 deaths were reported in the US [2]. The situation is predicted to further worsen by 2030 as the deaths due to cancer may cross 13.1 million [1]. The US net expenditure on cancer care is estimated to be shifted from 125 billion in 2010 to 156 billion by 2020 [2]. Seeing the tremendous increase in cancer incidences and prevalence worldwide, people have simultaneously developed anticancer drugs, but the cardiotoxicity caused by these drugs remained a major challenge for healthcare professionals [3]. Anticancer drug-related cardiotoxicity ranges from endothelial damage to myocardial infarction, angina, ischemia, cardiomyopathy and heart failure [4]. Understanding the molecular mechanism behind these unwanted side effects is of prime importance as it is directly related to the prognosis and survival rate of chemotherapeutically treated patients. A better insight into the intracellular signaling involved in the action of the anticancer drug will be instrumental in designing and developing novel drugs that will mitigate the unintended toxicities. Molecular basis of cardiotoxicity will also give an insight for the development of biomarkers that can be taken from preclinical study to pharmaceutical industries. Thus, in this review, we are highlighting the molecular mechanism of cyclophosphamide-induced cardiotoxicity which will be beneficial for the researchers in developing adjuvant therapy.

* Corresponding author at: Department of Pharmacology, Faculty of Pharmacy, Jamia Hamdard, New Delhi 110062, India.
E-mail address: sehaq@jamiahamdard.ac.in (S.E. Haque).

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2. Cyclophosphamide and cardiotoxicity

Cyclophosphamide is one of the potent alkylating agents used widely against rheumatoid arthritis, lupus erythematosus, multiple sclerosis, bone marrow transplantation, sarcoma, neuroblastoma, myeloma, lymphoma, ovarian cancer, lung cancer and breast cancer [5–7]. Cyclophosphamide is also a potent immunosuppressant drug. Thus it is used widely in organ transplantation and against graft rejection [7,8]. The historical aspect of alkylating agents go back to 2nd World War when the US released nitrogen mustard gas against the German air strikers in the Italian sea coast city Bari, in December 1943 [9]. This incident affected a few people leading to a tremendous reduction in WBC count [9]. After this incidence, secret studies were conducted in many research labs, including Yale University [10]. Gilman, Goodman, Lindskog, and Dougherty were the first ones to demonstrate the anticancer potential of mustard gas against lymphosarcoma [11]. There are many derivatives of nitrogen mustard used clinically now-a-days, and cyclophosphamide is one of them. Cyclophosphamide when administered, rapidly metabolizes into 4-hydrocyclophosphamide (4H-CYP) in the presence of cytochrome P450 isozyme and co-exists with its tautomer, aldophosphamide [12,13]. Aldophosphamide further interact with aldehyde dehydrogenase and forms carboxy-cyclophosphamide [12]. Aldophosphamide consequently decomposes into phosphoramide mustard & acrolein. Phosphoramide is an active neoplastic agent which acts on seven-guanine residues of DNA and causes tumor death, where as acrolein is a toxic metabolite that causes toxicity in the myocardium, cardiofibroblasts and endothelial cells [7,13] (Fig. 1). Cyclophosphamide exhibit the bimodal mechanism of antitumor action with cardiotoxic effect and immunomodulatory effects [14]. The difference in pharmacological effect depends on the metabolism of the drug, the dose administered, dosing schedule and timing of the dosing. [15]. CP or its metabolite exert an immunosuppressive effect at a lower dose (1–5 mg/kg, p.o) whereas higher dose (120–200 mg/kg i.v) exert cardiotoxic effect [16–19]. CP or its metabolite-induced immunosuppression affect both cell-mediated and humoral immunity. Lymphoid organs like bone marrow and thymus are primary organ for the formation and maturation of immune-competent T-cells. These mature cells then migrate from lymphoid organs to the peripheral organs where they elicit an immunological response or defensive function [20]. Any damage to these two organs would eventually lead to impaired immunocyte production and will result in immunosuppression (Fig. 1). On the other hand, spleen plays an important role in the colonization and elicitation of immune response. CP or its metabolite damages the bone marrow, thymus, and spleen leading to decreased lymphocyte count, B cells and T cells. [15,21]. Reduction in $CD3^+$, $CD4^+$, $CD8^+$ lymphocyte, macrophage activity, compromised IL-2, IL-6, 1L-10, INF-γ, TNF-α, IL-1α, altered Nrf2 and immunoglobulin A, G and M, have been reported upon cyclophosphamide administration [21]. Interestingly, it has been reported that all solid tumors contain infiltrate of leukocytes (myeloid and lymphoid lineage cells), neutrophils, mast cells, type 2-tumor-associated macrophage and T-regulatory cells [22]. These cells together prevent the cytotoxic potential of chemotherapy and thus protect the tumors from anticancer drugs. However, CP exhibits additional antitumor property by repressing the activity of the immune cell and thus increases the sensitivity of chemotherapeutic drug towards the tumor cells [23]. Cyclophosphamide and its metabolite like aldophosphamide, 4-hydroxy phosphamide and acrolein are considered as cardiotoxic. Out of these metabolites, acrolein is considered as the most cardiotoxic [12]. Acrolein is an unsaturated and highly reactive aldehyde, and cardiomyocytes are highly sensitive to this [24,25]. CP or its metabolite react with proteases and induces the oxygen free radicals generation and inflammation in cardiomyocytes [26]. CP or its metabolite also reduces the generation of endothelial nitric oxide synthase phosphorylation and reduces eNOS production. There are reports of CP mediated reduced eNOS dimer and increased eNOS monomer, leading to eNOS coupling that leads to the generation of nitrative stress by forming peroxynitrite (Fig. 1) [27].

Interestingly, a metabolite of CP, acrolein has striking chemical nature of forming cytoplasmic and nuclear protein adduct in cardiomyocytes, leading to cardiac damage [28]. Acrolein form adducts with lysine (aldanine) and react with glutathione and causes generation of oxidative stress. Acrolein also forms an adduct with cysteine (S-containing nucleophile) in cardiomyocytes, that leads to activation of caspase and NF-kB-p65 subunit [29]. Activation of caspases causes apoptosis whereas activated NF-kB translocate into the nucleus where it transcript the production of inflammatory cytokines like IL-6 and TNF-α. Formed adduct and metabolites of CP also interact with Fas ligand and TNF-α receptor (death receptor) and participate in cardiomyocytes apoptosis (extrinsic apoptosis) [27]. Protein adduct further damage cardiac mitochondria leading to diminished ATP production and induces subsequent cardiotoxicity. Direct damage to blood vessels, vasospasm and endothelial damage are also reported upon CP administration [24]. Another mechanism of CP or its metabolite- induced cardiotoxicity is activation of p53 & p38 mitogen-activating protein kinase pathways that causes cardiac apoptosis, inflammation and hypertrophy [30,31]. Additionally, activated p38 in cardiomyocytes upregulate the level of E3 ligase and Muscle RING-finger protein-1 (MuRF1), suggesting the evidence of myosin heavy chain degradation [28,32](Fig. 1). The recent finding also showed altered calcium homeostasis in cardiomyocytes that causes calcium overload, cardiac hyperactivity, hypertension, ER stress, depleted ATP production and increased sympathetic tone leading to cardiomyopathy and heart failure [24,33]. Therefore, it can be concluded that phosphoramide mustard is responsible for anticancer effect whereas immunosuppressant and cardiotoxicity is manifested by acrolein (Fig. 1). Acrolein-induced damage of thymus, spleen and bone marrow suppression causes immunosuppression. Cardiotoxic mechanism of CP or its metabolite consists of oxidative stress, nitrative stress, altered calcium homeostasis, formation of protein adduct that causes cardiomyocytes inflammation, apoptosis, cardiomyocytes swelling, nuclear splitting, vacuolization and alteration in signaling pathways like NFkB/p53/p38 MAPKs. These events lead to cardiomyopathy and heart failure and if remain untreated or undiagnosed, may lead to death.

3. Clinical perspective of cyclophosphamide-induced cardiotoxicity

Cyclophosphamide associated cardiotoxicity ranges from 7 to 28% [17,34]. Acute heart failure (HF) lies between 7 and 33% and is manifested within one week after the dose > 150 mg/kg, i.v. [17,35]. Available data supports that the mortality with CP ranges from 11 to 43%, which is generally observed within 7 to 21 days of its administration [17,35,36]. CP when given in dose range of 170–180 mg/kg, i.v for 4–7 days, acute cardiotoxicity was observed in 22% of patients whereas 11% of patients developed fatal cardiotoxicity [37]. In another study when cyclophosphamide was given at the dose of 200 mg/kg, i.v for four days, it resulted into the development of cardiotoxicity as manifested in 25% of the patients [17].

It is well established that significant heart function problems are known side-effects of cyclophosphamide administration when high doses of the drug is given to humans or animals. High-dose of cyclophosphamide is well-established and widely used in sequential high-dose chemotherapy (HD-CHT) protocols for solid and hematological malignancies, autoimmune disorders like systemic sclerosis, multiple sclerosis and aplastic anemia as well as in transplant conditioning regimens. [36–41] The cyclophosphamide at the dose 200 mg/kg, i.v., or above is used in bone marrow transplantation whereas dose of 80–200 mg/kg, i.v., is used in the patients of solid tumor and hematological malignancies [16,17,42]. The rationale for using dose of 200 mg/kg, i.v., is based on the previous findings where relapse of disease was found at the dose of 180 mg/kg, i.v. [38,43–45] Therefore,

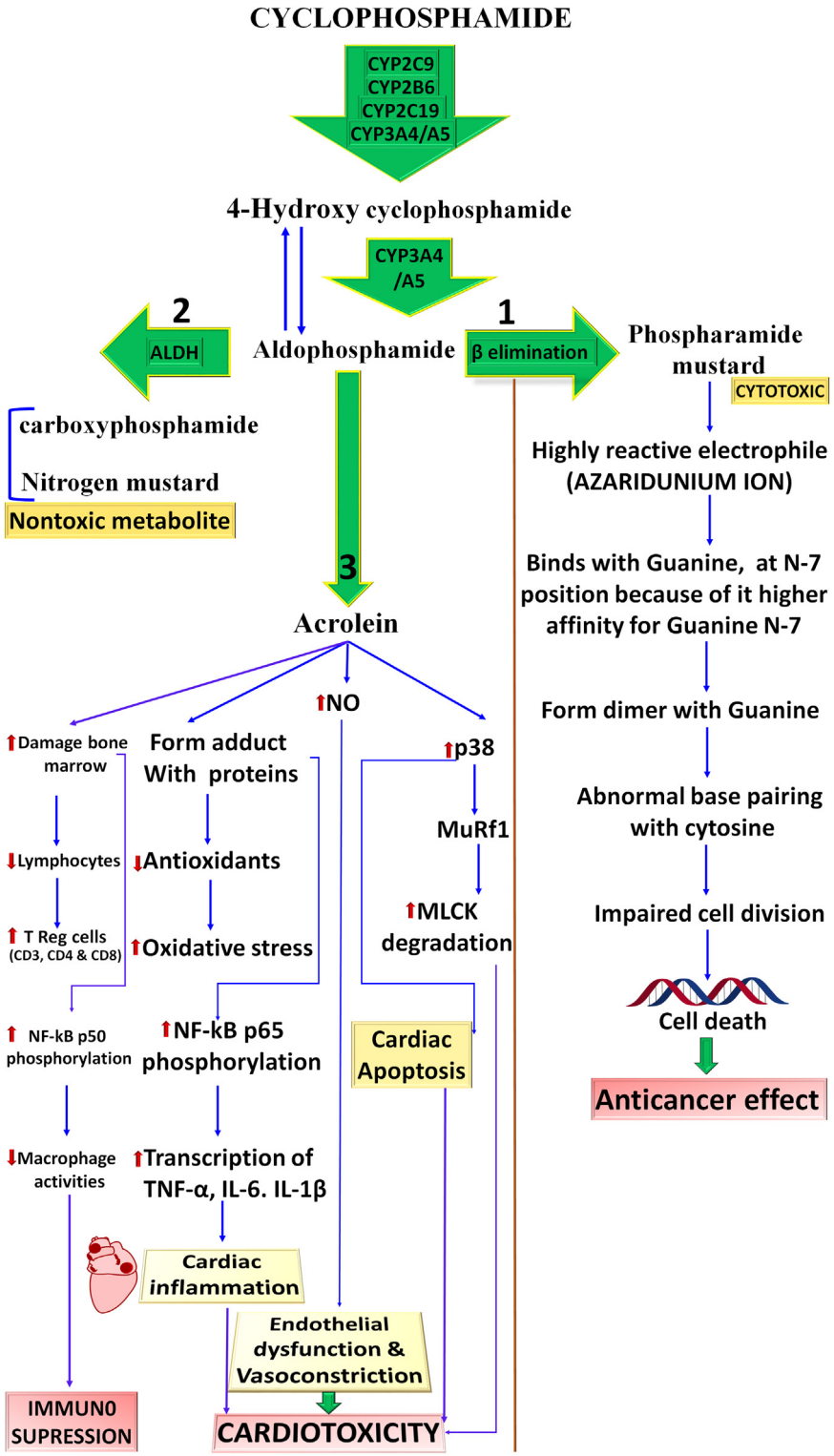

Fig. 1. Showing metabolism of Cyclophosphamide (CP). CP undergoes hepatic metabolism and forms 4-hydroxy cyclophosphamide (4-HCY) which further metabolizes into aldophosphamide. Aldophosphamide produces phosphoramide mustard via β-elimination which show anti-cancer activity. Aldophosphamide further metabolizes into non-toxic metabolites like carboxyphospamide and nitrogen mustard along with toxic compound acrolein which is mainly responsible for CP induced toxicities.

it was concluded that cyclophosphamide at the dose of 200 mg/kg, i.v. has the ability to amply supress the immunity and facilitate engraftment [40]. Although dose higher than the 200 mg/kg, i.v has been reported to cause cardiotoxicity, but depending on the merit of the clinical outcome, higher dose of CP has been widely used in many cases, like in refractory neuromyelitis optica spectrum disorder, (200 mg/kg, i.v.), in non-seminomatous germ cell tumor in the testicle, (235 mg/kg, i.v), and in rapidly progressive systemic sclerosis (SSc) with diffuse skin and visceral involvement, (300 mg/kg, i.v.) were used [41,46].

However, as far as predictive dose of CP or its metabolite to act as a point of cardiotoxicity is concerned, we encountered a variable range of doses rather than a single dose. Thus, it is quite difficult to predict

which patient and at what dose would be susceptible for cardiotoxicity. Based on the literature review and case reports, it was found that the total dose of CP administered, the age of patients, and prior history of medication are well-defined risk factor for cardiotoxicity [47,48]. Santos et al., 1971, first time reported the cardiotoxicity at the dose range of 200 to 270 mg/kg, i.v., Mills and Roberts 1979, have reported cardiotoxicity at the dose of 144 mg/kg, i.v. and 168 mg/kg, i.v [44]. Gottdiener et al., and Steinherz et al., 1981, had reported cardiotoxicity at the dose > 80 mg/kg (range 80–200 mg/kg, i.v. for solid tumors and before bone marrow transplant) [16,42]. Kamezaki et al., 2005 reported cardiotoxicity at the dose of 100 mg/kg, i.v. in allogeneic stem cell transplant whereas Katayama et al., 2009 has reported fulminant fatal congestive heart failure in diffused large B cell lymphoma at the dose of 75 mg/kg, i.v [45,49]. This was the lowest reported dose exhibiting cardiotoxicity. Recently Park et al., 2018 had reported cardiotoxicity at the dose of 120 mg/kg, i.v. when used for the treatment of allogeneic hematopoietic stem cell transplantation [38]. However, based on the clinical studies, CP at the dose of 120–200 mg/kg, i.v. or above, is considered as cardiotoxic [16–19,44,50].

Importantly, term 'cardiotoxicity is often used to describe the toxicity of heart. Cardiotoxicity can be defined as pathological state where heart muscles get weaker and unable to pump sufficient blood to the body, leading to cardiomyopathy under the influence of toxic substances like doxorubicin, cyclophosphamide, 5 FU, tyrosine kinase inhibitors or isoproterenol [51–53]. Cardiomyopathy is a clinical manifestation of cardiotoxicity where cardiac chambers become stretched (dilated cardiomyopathy), thickened/enlarged (hypertrophic cardiomyopathy) or stiff (restrictive cardiomyopathy) [46,54]. According to the US National Cancer Institute, term cardiotoxicity is defined as "toxicity that affects the heart" [55,56]. This definition includes the direct effect of the cardiotoxic agent on the entire cardiovascular system and also an indirect effect on cardiac cells due to the alteration in hemodynamic flow or alteration in the thrombogenic event that causes coronary artery disease and subsequent damage to the cardiac cells [57]. According to this definition, cardiotoxicity encompasses multiple clinical manifestations such as hypertension, myocardial ischemia, myocardial infarction, valve damage, pericardial disorder, arrhythmia, congestive heart failure, cardiomyopathy and heart failure [56]. It is important to bring in notice that there is no universal definition of cardiotoxicity, but American society of cardiology has defined 'cardiotoxicity due to chemotherapy' as coronary artery disease, cardiac arrhythmia, electrical conduction dysfunction and heart failure [58] American Society of Echocardiography and European Association of Cardiovascular Imaging and Cardiac Review and Evaluation Committee has defined "cardiotoxicity" as heart failure and decline in LVEF from > 10% to < 55%, measured by echocardiographic like multigated acquisition (MUGA) scan, and 2D or 3D contrast cardiac MRI [59]. Apart from the radiological examination of cardiotoxicity, prediction of myocardial strain or myocardial damage by troponin I is a potential as well as a promising serum marker of cardiotoxicity [60].

3.1. Dose regimen of cyclophosphamide in various diseased condition

Cyclophosphamide is used in different types of solid tumors, hematological malignancy, nephrotic syndrome and in bone marrow transplantation. In the induction therapy for the malignant disease, intravenously CP is used at the dose of 40 to 50 mg/kg over 2–5 days, whereas orally at the dose of 1–5 mg/kg. For the maintenance therapy, CP is used at the dose of 10–15 mg/kg i.v over 7 to 10 days or 3 to 5 mg/kg twice weekly. In children, for the treatment of nephrotic syndrome, CP is used orally at the dose of 2–3 mg/kg for 2–3 month. For the treatment of stem cell transplant, it is used at the dose of 120 to 300 mg/kg, i.v. over 2–4 days [38,41]. For the treatment of systemic sclerosis i.v. dose up to 300 mg/kg have been used over four days whereas for the treatment of systemic lupus erythematous (SLE) initial or a standard dose of 15 to 30 mg/kg i.v. is used monthly, followed by same dose quarterly for two years. In case of persistent SLE, CP is used at the dose of 200 mg/kg, i.v. over four days [61,62]. Use of CP at the dose of 200 mg/kg over four days is also reported for the treatment of rheumatoid arthritis, refractory autoimmune hemolytic anemia, refractory myasthenia, aggressive multiple sclerosis without bone marrow transplantation, pemphigus vulgaris, Wegener granulomatosis, Vasculitidies, Wegener granulomatosis and autoimmune enteropathy [63–66].

3.2. Combination of cyclophosphamide with other anticancer drugs, their mechanism of cardiotoxicity and cardiotoxic sequalae

Cyclophosphamide is commonly used in combination with other chemotherapeutic agents in the clinic (e.g., cyclophosphamide, methotrexate, and fluorouracil (CMF) in the treatment of some breast cancers [67,68]. In non-Hodgkin's lymphoma CHOP and R-CHOP regimen is used (cyclophosphamide, doxorubicin, vincristine (Oncovin) prednisone and rituxan) [69]. BEACOPP regimen (bleomycin, etoposide, doxorubicin, vincristine, procarbazine, and prednisone) and EPOCH/R-EPOCH (rituximab, etoposide phosphate, prednisone, vincristine (Oncovin), cyclophosphamide, and doxorubicin) is commonly used in Hodgkin's lymphoma [70,71]. For the treatment of multiple myeloma VBMCP regimen (vincristine, carmustine, melphalan, cyclophosphamide, and prednisone) is used [72]. VAC regimen (vincristine, actinomycin, and cyclophosphamide) is used in the treatment of metastatic urothelial cancer, childhood soft tissue sarcomas and ovarian germ cell tumor [73]. Some of the combined regimen used in the treatment of breast cancer is as follow (1) CMF (cyclophosphamide, methotrexate, and 5FU) (2) cyclophosphamide with doxorubicin and with or without tamoxifen in stage II node-positive cancer and (3) cyclophosphamide, melphalan and prednisoneare used in stage III of advanced cancer. (4) FAC regiment (5FU, cyclophosphamide, and doxorubicin). For the treatment of the lungs cancer, CAV regimen (cyclophosphamide, Adriamycin, and vincristine) is used [74]. Apart from above mentioned combined regiment, selection of combined drug vary, depending upon the types and severity of disease, duration of treatment, previous history of treatment/radiation and state of the patient.

Further it is important to discuss that CP when administered alone, is often used in metronomic dosing, that is, using multiple application of sub-toxic concentration over repeated period of time to achieve clinical benefits and to mitigate the toxicities. However, depending on the disease condition, CP is also used in combination with other anticancer drugs at high doses and in bolus dosing schedule, as discussed in the previous section [63–66]. There have been reports of synergistic cardiotoxic effects while using CP in combination with anthracyclines, human epidermal growth factor receptor 2 (HER-2) antagonists, tyrosine kinase inhibitors (TKIs), platinum analogue, antimetabolites andtaxens. Doxorubicin induces cardiotoxicity by generating semiquinone radicals; cyclophosphamide induces cardiotoxicity by direct damage to cardiac cells and endothelium by the generation of acrolein [75]. HER-2 antagonist like trastuzumab interferes with angiogenesis and repair mechanism of cardiac cells. TKIs like gefitinib, erlotinib, lapatinib, canertinib, semaxinib, vatalanib, sorafenib, and leflunomide inhibit vascular endothelial growth factor (VEGF) leading to reduced eNOS generation and hypertension [75–77]. Platinum analogue like cisplatin-induced cardiotoxicity is the result of a platinum generation that damage endothelium and increases carotid intima-media thickness. Therefore, it is evident that different anticancer drug exhibit different mode of cardiotoxicity but they have similarity in exerting cardiac manifestations. CHF, arrhythmia, LVDF, tachycardia, and MI are exhibited by doxorubicin, cyclophosphamide, paclitaxel, 5-FU, rituximab, trastuzumab, etoposide, vincristine and cisplatin whereas busulfan primarily causes endocardial fibrosis, paclitaxel causes arrhythmia. 5FU, etoposide and cisplatin cause damage to blood vessels leading to coronary artery disease [78]. Combination of trastuzumab, with

doxorubicin, cyclophosphamide, taxens, paclitaxel are considered as one of the most cardiotoxic combinations as blockage of HER-2 receptors by HER-2 antagonist prevent the recovery or repairing capacity of cardiac cells, damaged by the anticancer drugs. [76,77]. Combination of tyrosine kinase inhibitors (TKIs) with cyclophosphamide, trastuzumab, 5-FU or cisplatin aggravate the cardiotoxicity as TKIs reduces the production of eNOS resulting into hypertension and endothelial dysfunction. TKIs are also VEGF blocker so prevent the angiogenesis. Therefore, there is potentiation of vasoconstrictive effect, endothelial dysfunction as well as cardiac damage that failed to regenerate and survive leading to hypertension or HF [78,79]. Combination of cisplatin with cyclophosphamide or with 5-FU aggravates cardiotoxicity as platinum analog primarily damage the kidney leading to hypomagnesia and hypokalemia that results in hypertension. Cisplatin also predisposes myocardium to significant arrhythmia, and this situation is aggravated when administered with cyclophosphamide [80]. Combination of Texans like paclitaxel with cyclophosphamide or doxorubicin causes cardiac damage as paclitaxel delays the elimination of these anticancer drugs. Paclitaxel also causes fluid retention so aggravate the situation of CHF [81]. FDA warranted the precaution upon using methotrexate with cyclophosphamide. CMF regimen, commonly used in breast cancer is reported to cause cardiotoxicity [82]. The cardiotoxic event consists of pericarditis, myocardial hemorrhage exhibited by cyclophosphamide whereas 5-FU causes direct damage to blood vessels and endothelium and methotrexate causes arrhythmia [83,84]. One of the most common drugs used in the treatment of cancer and other autoimmune disorder is corticosteroid (prednisone). Cortisol has been reported to cause hypertension, hyperlipidemia, and can accelerate coronary artery disease. Cortisol is also used with cyclophosphamide and melphalan in the management of pain associated with breast cancer and in stage III advance cancer [85]. Independent of its cardiotoxic effect, cortisol is known inducer of cytochrome P450 isozymes (CYP 3A4, CYP2B9). These isozymes stimulate the hepatic metabolism of cyclophosphamide and enhance the production acrolein that involves in cardiotoxicity [86]. Thus, there is the direct potentiation cardiotoxic effect of cortisol with cyclophosphamide.

There are clinical findings of short-term and long-term follow up studies, metanalysis, and systemic review which confirm the cardiotoxic sequalae of cyclophosphamide and other related drugs. Beside this, one of the studies however, have reported that cyclophosphamide is not associated with any cardiotoxic sequalae [16]. On the other hand, a number of studies have reported the cardiotoxic sequalae of cyclophosphamide, since cyclophosphamide exhibit acute as well as chronic cardiotoxicity which is reflected over 1–3 weeks of drug administration or even, after years of the drug administration. [17,48,87–90].

One of the oldest and longest follow up study by Makinenet al., 1990, had reported the persistence cyclophosphamide-induced cardiotoxic sequelae. This was 10.3–27.34 year follow up study where the patient who underwent cyclophosphamide exposure exhibited abnormal QRS, PQ interval, and depressed ST interval leading to heart failure [91]. Another study by Klein et al., 2000, where 105 patients were followed up for two years with stage II/III breast cancer or stage IV metastatic breast cancer, patients were administered with doxorubicin and paclitaxel chemotherapy followed by a high dose of cyclophosphamide [92]. Cardiac sequelae reported in this case were reduced LVEF, vasoconstriction and congestive cardiac failure. Similarly, 5-FU, monoclonal antibodies, and tyrosine kinase inhibitors have been reported with cardiotoxic sequelae. Sawaya et al., 2012, had reported cardiotoxic sequalae when the patients of breast cancer were exposed to anthracycline therapy, followed by trastuzumab and taxanes. This finding was the result of 15 months follow up where after 15 months of dose free period, the patient had reduced LVEF, persistent circumferential myocardial strain, reduced ejection fraction and elevated tropT and BNP. 32% of patients had been diagnosed with HF after 15 months [93]. Similarly, a prospective blinded observational study reported the cardiotoxic sequalae with anthracycline after three years of treatment. 59% of total patients have been reported with LVEF and CHF [94].

3.3. Scientific and clinical significance of thoroughly documented changes in cardiac myocytes

Cardiotoxicity due to CP is manifested at the high dose and is considered as one of the complications of a bone marrow transplant [16,95]. Administration of a high dose of cyclophosphamide results in acute as well as chronic cardiotoxicity [4,54,95]. The exact pathogenesis of cardiotoxicity is still not known; however, it is thought to involve direct damage to endothelial and cardiomyocytes. Findings showed left ventricular wall thickness, and thickness of interventricular septum due to interstitial edema that causes left ventricular diastolic dysfunction, increased myocardial echogenicity, pericardial effusion, and reduced systolic function leading to cardiomyopathy and heart failure [45,96,97]. Cyclophosphamide has been reported with ischemic damage because of intrapapillary microemboli. The histological findings indicate acute pericarditis and myocardial hemorrhage, atrophic & focal necrosis with interstitial edema ischemic myocardial ischemia [19,45,98]. Ultrastructural examination also showed the multifocal myocardial necrosis with microthrombi in capillaries, fibrin strands in the interstitial, disrupted and aggregated mitochondrial cristae and degenerated myocardial tissue. The overall cardiotoxic effect comprises of CHF, cardiomyopathy, cardiac tamponade, heart failure, and death [98].

Clinically, ECG, ultrasound cardiography, (UCG), Multigated Acquisition Scan (MUGA), 2D or 3D contrast cardiac MRI and serum markers like TropT and BNP are used to detect and predict early phase cardiotoxicity [98]. Till date, there is no clinically approved therapy available to prevent the cardiotoxic effect of cyclophosphamide. At the same time, mesna a detoxifying agent, used with the cyclophosphamide to reduce its bladder toxicity has been reported with altered QT and ST cardiotoxic effect. In patients with or without previous history of cardiovascular dysfunction, screening using novel technique like MUGA scan, 2D or 3D MRI for left ventricular ejection fraction (LVEF) is performed [90]. However, measurement of an early peak flow velocity to atrial peak flow velocity (E/A), deceleration time (DT), isovolumetric relaxation time (IVRT) and serological testing could also be a better approach to diagnose and treat acute cardiotoxicity which is not reflected in radiological examination [90,99]. Additionally, other than monotherapy of cyclophosphamide, multitherapy with chemotherapeutics or radiation therapy, it becomes imperative to consider the potential interaction between the treatment [90]. In the animal, it has been reported that administration of cyclosporin with cyclophosphamide prevents the cardiotoxicity, (Fig. 8) support the promising role of altered mitochondrial permeability, apoptosis and calcium dysfunction in cardiotoxicity. However, clinically there are no reports of protective therapies for CP-induced cardiotoxicity [98]. Therefore, the establishment of onco-cardio clinic could be the best approach to keep pace with the cancer therapies and the magnitude, incidence, and consequences of their cardiovascular side effects.

4. Preclinical perspective of cyclophosphamide-induced cardiotoxicity

4.1. In-vitro evidence for cyclophosphamide-induced cardiotoxicity

Cardiotoxic side effect of cyclophosphamide is a major challenge. Till date, pathophysiology of cyclophosphamide-induced cardiotoxicity is not clearly understood [7]. CP is known to exert cardiotoxicity but this cardiotoxicity is exerted only after its metabolism. [12,100]. In the in vitro study effect of different metabolites of CP on H9C2 cells was demonstrated using H9C2 cells. Cells were exposed to various CP metabolites like 4-hydroxy-cyclophosphamide (HCY), acrolein and carboxyethyl phosphoramide (CEPM). MTT assay was used to evaluate the extent of cardiotoxicity by measuring the level of LDH and ROS. In this

study, level of aldehyde dehydrogenase (ALDH) was also determined, because study conducted by Nishikawa et al., 2016, it was found that ALDH is responsible for increasing the level of CEPM. The outcome of the study not only showed that CP is not associated with cardiotoxicity but metabolite like CEPM also manifested no cardiotoxic effect [100]. 4-HCY as well as acrolein which get formed from aldophosphamide after two hours of exposure found to be cardiotoxic but acrolein was found comparatively more cardiotoxic than HCY. In vitro study also showed that exposure to N-acetylcysteine (NAC) attenuated the CP-metabolites related cardiotoxicity [100,101]. An important conclusion drawn from the above discussed study was the role of Aldophosphamide that metabolizes to CEPM in the presence of aldehyde dehydrogenase [101]. Downregulation of ALDH retards the formation of CEPM and stimulates the formation of acrolein. Interestingly, Ren et al., 1997, had also reported the inhibitory effect of acrolein on ALDH activity [102]. Based on the above discussed study, it can be concluded that CP metabolites mainly 4-HCY and acrolein are responsible for cardiotoxicity whereas CP alone and another metabolite, CEPM was found to be non-cardiotoxic. Increasing the activity of ALDH and reduction in ROS generation could be a therapeutic approach to mitigate cyclophosphamide-induced cardiotoxicity.

4.2. In vivo prospective of cyclophosphamide-induced cardiotoxicity

In the previous section we have discussed the clinical and in vitro perspective of CP-induced cardiotoxicity. This section deals with the in vivo aspect of cardiotoxicity in animal models. Preclinical studies play a vital role in understanding the effect and mechanism of action of certain drugs. Detailed molecular mechanism of CP-induced cardiotoxicity based on preclinical study (in vivo studies) has been discussed below.

4.3. Cyclophosphamide and energy pool alteration

For the optimum working of the heart, cardiomyocytes require a continuous supply of energy in the form of ATP [52,103]. Fatty acid and glucose are two common substrates for cardiomyocyte energy production [52]. Long chain fatty acid (LCFA) oxidation in cardiac muscles differs significantly from the rest of the organs. In a normal adult's working heart, LCFA oxidation accounts for > 60–90% of ATP production [104,105]. Rest of the ATP is produced from glucose, lactate and ketone bodies [104,105]. There are two important key regulators of fatty acid metabolism (i) heart fatty acid binding protein (H-FABP) and (ii) carnitine palmitoyl transferase-I (CPT-1) [106]. H-FABP is found in the cytosol of cardiomyocytes and plays an important role in transporting fatty acid to mitochondria [107]. This transportation firstly provides a substrate for energy production and secondly, its regular transportation removes the free fatty acid (FFA) and their toxic intermediates from the cytosol [107]. CPT-1 or carnitine acyltransferase-1 however, is accountable for the activation and transportation of LCFA across the mitochondrial membrane in cardiomyocytes [105,108]. CPT-1 causes transesterification of the fatty acyl group to form fatty acyl-carnitine, which crosses inner mitochondrial membrane and reaches the matrix where it gets converted back into fatty acyl CoA by CPT-II and then enters into β-oxidation to produce ATP (106 equivalent to 3233 kJ/mol for palmitate) [109]. Any alteration in the level of these key molecules results in significant cardiac dysfunction and cardiomyopathy as seen with various studies which established that CP, doxorubicin and other anticancer drugs alter the expression of H-FABP & CPT-1 [105,110,111]. CP-induced downregulation of H-FABP mRNA is reported to cause inhibition of fatty acid oxidation [105]. This inhibition results in the accumulation of FFA and toxic intermediate in the cardiac cytosol and diminishes the ATP production [105]. In the doxorubicin model, administration of carnitine significantly attenuated the cardiotoxicity by up-regulation of H-FABP & CPT-1 mRNA, whereas carnitine deficiency was reported to aggravate cyclophosphamide-induced cardiotoxicity [105,112]. Acetyl CoA carboxylase (ACC), malonyl CoA and malonyl CoA decarboxylase (MCD) are three functional enzymes that in collaboration with CPT-1 regulate the LCFA oxidation [105]. ACC is the primary enzyme responsible for the synthesis of Malonyl CoA through irreversible carboxylation of acetyl CoA [105]. Increased malonyl CoA inhibits the CPT-1 mediated LCFA activation in the mitochondria [105,110,111]. ACC enzyme is found in two isomeric forms, ACCa/ACC-1,andACCb/ACC-2.ACCa/ACC-1 is present significantly in the lipogenic tissue whereas ACCb/ACC-2 is found in skeletal tissue and theheart [113]. Earlier studies have shown that CP causes a reduction in CPT-1, increase in ACCb/ACC-2 and malonyl-CoA mRNA expression [105,110,111]. It is well known that MCD carries out the decarboxylation of malonyl-CoA that results in the formation of acetyl CoA. Therefore, the optimum level of MCD is beneficial for normal working of heart [104,105,113]. Recent studies have shown that the reduced expression of MCD mRNA in CP administered animal, the cardiotoxic effect of malonyl CoA and reduced the cardioprotective effect of CPT-1 [105]. Further, it is apparent that CP downregulates the function of H-FABP, CPT-1, and MCD and up-regulates the function of ACCb/ACC-2 and malonyl CoA. The ultimate effect results in the inhibition of LCFA oxidation and diminished ATP production. When cardiac tissue is deprived of enough ATP, it alters contraction and relaxation, accumulates calcium in mitochondria and causes endoplasmic reticulum stress [114,115]. Reduced ATP also leads to the failure of Na^+-Ca^{++}pump, Ca^{++}ATPase activity, and Na^+-ATPase activities which leads to the elevated intracellular calcium concentration in cytoplasm or sarcoplasm [115]. Increased intracellular calcium thus carries out the diverse pathological functions like altered cardiac contraction, cardiomyocytes inflammation, apoptosis, and induces production of reactive oxygen species (ROS) & reactive nitrogen species (RNS)resulting into cardiomyopathy, cardiac hypertrophy and heart failure (Fig. 2) [116].

4.4. Cyclophosphamide and oxidative stress

One of the major causes ofcardiotoxicity induced by cyclophosphamide is the oxidative stress [7]. Oxidative stress is defined as an alteration in the level of intrinsic anti-oxidants, abnormal production of ROS & RNS [35] CP has been reported to induce oxidative stress in cardiomyocytes by decreasing the antioxidant level. Based on the preclinical studies, cyclophosphamide when administered at a single dose of 150–200 mg/kg i.p. resulted into the development of oxidative and nitrative stress within a week [117]. Recently, a published data showed that the administration of CP at a dose of 50 mg/kg, i.p. for three consecutive days resulted into the development of oxidative stress [7]. Oxidative stress maybe triggered by mitochondria or endoplasmic reticulum and involve NADPH, ROS, RNS and Nrf-2-OH signaling pathways. Effect of CP on oxidative stress has been discussed below (Fig. 3).

4.4.1. Mitochondrial dependent oxidative stress

Mitochondria are one of the most commonly affected cell organelles in CP-induced cardiotoxicity. CP or its metabolite causes swelling of mitochondria, aggregation of chromatin, condensation of cytoplasm, splitting and vacuolization of the nucleus [118,119]. It is reported that CP administration causes downregulation of fatty acid oxidation in cardiac mitochondria and upregulation of glucose metabolism [105]. This paradigmatic shift is more often a reported feature of the heart failure [104]. Since > 90% of ATP is produced from mitochondria [115,120], any changes in mitochondria or electron transport chain may aggravate myocardial damage. Further, mitochondrial damage diminishes the net ATP production that weakens the myocardial cells. Additionally, downregulation of ATP production followed by mitochondrial damage alters the calcium homeostasis [114,115]. Altered calcium regulation then initiates the malfunctioning of cardiomyocytes, increases nitrite stress, calpain level, stimulates inflammatory cascade and triggers apoptosis [114,121]. Recently thymoquinone has been reported to possess cardioprotective potential against

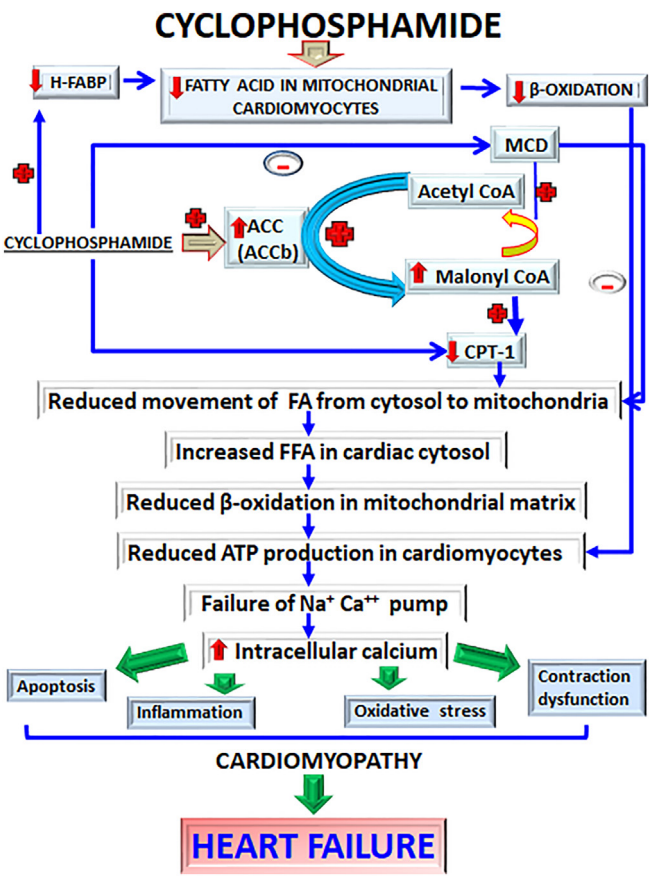

Fig. 2. Schematic diagram showing the effect of cyclophosphamide (CP) on fatty acid (FA) metabolism in the heart. CP downregulates the heart fatty acid binding protein (H-FABP) resulting in the reduced transportation of free fatty acid (FFA) from the cytosol to mitochondria. It further participates in cardiotoxicity by potentiating the downregulation of carnitine palmitoyltransferase I, increasing the activity of Acetyl CoA carboxylase (ACCb) which causes carboxylation of acetyl CoA, and forming malonyl-CoA. Malonyl-CoA is known for downregulation of CPT-I and malonyl CoA decarboxylase (MCD) for decarboxylation of malonyl-CoA toAcetyl CoA. Thus reduced MCD level causes enhanced malonyl-CoA activity and diminished CPT-I activity, leading to decrease in β oxidation.

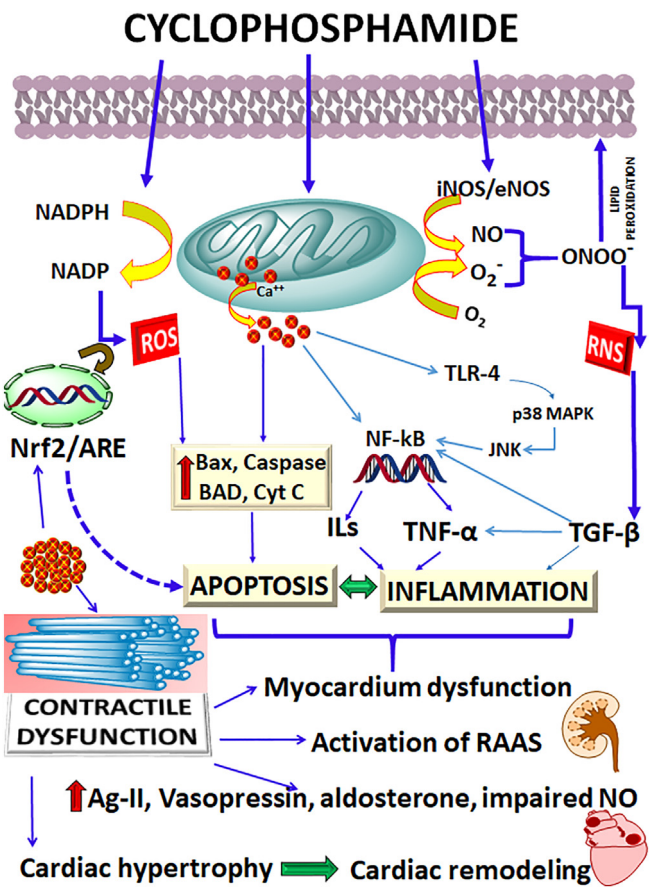

Fig. 3. Schematic diagram showing the generation of cyclophosphamide (CP) induced oxidative stress and nitrative stress. Oxygen free radicals combine with nitrogen free radicals to generate peroxynitrite (RNS). Stress condition triggers the accumulation of intracellular calcium which mediates apoptosis via BAX/Caspases. Stress stimuli also initiate the Toll-like receptor-4 (TLR-4) mediated activation of p38 and c-Jun N terminal kinase (JNK). Calcium accumulation leads to the activation of NF-kB that further increases the expression of pro-inflammatory cytokines (TNF-α and ILs). Nitrative and oxidative stress collectively activates TGFβ which suppresses Nrf2/ARE signaling pathway. The oxidative and nitrative stress-mediated apoptosis and inflammation eventually lead to cardiac hypertrophy and cardiac remodeling.

cyclophosphamide-induced cardiotoxicity [122]. Beside thymoquinone, glutathione, crocin, *N*-acetyl cysteine, probucol, lupeol, blueberry anthocyanin, and kolaviron have shown cardioprotective action against CP-induced cardiotoxicity via combating the oxidative stress [7,123–128]. These bioactive molecules cause overexpression of endogenous antioxidant enzymes that directly reduces hydrogen peroxide and protect cardiomyocytes. Therefore, we can say that CP-causes an alteration in the mitochondrial-mediated metabolic process as well as participates in the progenesis of pathological conditions. Role of various signaling cascades, inflammatory and apoptotic mediators and calcium dysfunction have been shown in Fig. 3.

4.4.2. NADPH production and reactive oxygen species (ROS)

CP is associated with free radical generation [7,129] which are produced by enzyme systems like mitochondrial NADPH, NADH dehydrogenase and NADPH oxidase [130]. Based on previous studies, it was reported that cardiomyocytes when exposed to CP, results into elevation of NADPH oxidase and other ROS mediators [129–131]. Apart from cyclophosphamide, doxorubicin, isoproterenol, and arsenic trioxide are also reported to generate oxidative stress via NADPH pathways [132,133]. NADPH mediated oxidative stress is further reflected into an alteration of Nrf2-HO/Nrf2-NQO-1 pathway that finally leads to cardiotoxicity [134] (Fig. 3).

4.4.3. Nrf2 and oxidative stress

Nrf2 is a basic leucine zipper protein which is actively involved in the regulation of antioxidants. Literatures provide increasing evidence for the down-regulation of Nrf2 followed by CP administration [134,135]. In one of the studies, single i.p. injection of cyclophosphamide administration at the dose of 200 mg/kg, resulted into the reduction of Nrf2 expression, increase in DNA fragmentation and myocardial inflammation. Pretreatment with thymoquinone at the dose of 5, 10 and 20 mg/kg, i.p. for eight days resulted into a significant increase in Nrf2 and attenuated the DNA fragmentation and cytokines generation [135]. However, a contrast observation was reported by Tripathi et al., 2010, where they conducted two sets of experiment by administering CP after and before the test drug, i.e., following pretreatment and post treatment regimen. They found a contrast behavior of Nrf2 in these two cases. In the pretreatment regimen, where CP was given on the 4th day & sacrificed on 5th day, Nrf2 expression was increased, whereas in the post treatment regimen where CP was given on 1st day and scarifies on 14th day, Nrf2 expression was decreased [134]. They have attributed to this behavior as a self-compensatory adaptation of the body where Nrf2 increases in response to CP administration.

4.4.4. Lipid peroxidation and oxidative stress

Lipid peroxidation is a chain reaction, initiated by reactive oxygen species that influences unsaturated fatty acid in the cell membrane, lipoproteins and other molecules that contain lipids [136]. Once the lipidis peroxidized, it alters the membrane permeability and integrity [136]. Studies have shown that CP when administered at a dose of 75 mg/kg, i.p. in a pretreatment regimen resulted into lipid peroxidation with a reduction in endogenous antioxidant enzymes. In the same experiment, selenium pretreatment restored the lipid bilayer structural integrity and caused a marked increment in antioxidant enzymes [136]. Recently Bhatt et al., 2017, experimented with post-treatment regimen of cyclophosphamide at the dose of 200 mg/kg, i.p. and used mangiferin as a test drug. [137]. CP treated group showed marked oxidative stress and lipid peroxidation, whereas mangiferin treated group augmented the CP-induced lipid peroxidation and restored antioxidant level [137]. It is important to understand the fact that along with ROS, intracellular calcium plays a significant role in lipid peroxidation [114,138]. Lipid peroxidation followed by the action of phospholipids A2 leads to the formation of arachidonic acid (AA) (Fig. 3). Cyclooxygenase-2 (COX-2) acts on the formed AA and produces prostaglandin [117]. Now, these prostaglandins induces inflammatory cascade that results in myocardial damage (Fig. 3). Lipid peroxidation is not only accountable for the generation of prostaglandins, but also for inflammation and apoptosis [139]. ROS and RNS contribute to alteration of Nrf2 signaling along with the increased intracellular calcium [139]. Calcium directly activates proapoptotic genes like Bax and BAD [140,141]. Calcium also participates in the activation of TLR-4, a well-known protein of the inflammatory cascade leads to the initiation of signaling pathway [141,142]. TLR-4 activates and increases the expression of NF-kB via p38 MAP Kinase and JNK pathway [141–143]. On the other hand, NF-kB mediates the formation of pro-inflammatory interleukins and TNF-α [141] (Fig. 3). Available literature confirms that the downregulation of TLR-4 attenuate anticancer drug-induced cardiomyopathy, inhibit cardiac fibrosis and suppresses cardiac inflammation [144–146]. Thus, we can hypothesize that blocking the action of TLR-4 could be a powerful weapon in the mitigation of CP induced cardiotoxicity.

4.5. Cyclophosphamide and endoplasmic reticulum stress (ER stress)

Pathological condition induced by a toxic substance or toxic metabolite like acrolein always induces myocardial endoplasmic reticulum stress [24]. Literature supports the finding that ER stress is directly associated with apoptosis [147] (Fig. 4). Beside ER stress and apoptosis there are evidence for impaired calcium homeostasis which is mediated by alteration in sarcoplasmic reticulum Ca^{++} ATPase (SERCA2a), phospholamban (PLB) and calsequestrin [148–151]. As discussed in the previous section, CP causes significant oxidative stress, and mitochondrial damage, the probability that the endoplasmic reticulum would be under stress in such a situation is always high [152]. These changes collectively results in systolic and diastolic dysfunction.

4.6. Cyclophosphamide and nitric oxide

Cardiotoxic role of nitric oxide by the anti-neoplastic drug has been confirmed by several studies [153]. CP administration has been reported with up-regulation of NO that can be because of the action of iNOS or eNOS [153–158]. Discussing the exact role of iNOS/eNOS in the pathogenesis of cardiac dysfunction is controversial [153,159]. There are studies which have reported negative as well as positive effects of iNOS/eNOS in animal models [160,161]. Further, increased or decreased level of iNOS/eNOS induced cardiotoxicity vary from model to model [153,159]. We conclude that the cardioprotective effect of iNOS/eNOS is because of its NO generation capacity, whereas a cardiotoxic impact is due to the generation of peroxynitrite ($ONOO^-$) (Fig. 5). Peroxynitrite is formed when NO reacts with oxygen free radicals (O_2^-) [162]. iNOS and eNOS have a significant role in cardiomyopathy, however eNOS has a double role (depending upon O_2^- concentration) whereas iNOS has a cardiotoxic effect in general. During CP-induced toxicity there is an increased in the level of iNOS and NO leading to nitrative stress which is accomplished by the presence of reactive oxygen species [159]. Nitrative stress further regulates the apoptotic pathway via p38/JNK cascade [163].

4.7. Cyclophosphamide and cardiac inflammation

Cardiac inflammation is a pathological condition which is observed after exposure to a cardiotoxic agent. It is well established that the NF-kB signaling pathway is crucial for the formation of pro-inflammatory cytokines [6]. The IκB kinase (IKK) complex consists of ikk-α & ikk-β, both of which are the key regulators of NF-kB activation. IkB-α is an inhibitory protein that inhibits the transcription factor of NF-kB. This phenomenon causes sequestration of NF-kB in the cytoplasm [164]. In brief, ikB-α keeps NF-kB inactivated in the cytoplasm and thus inhibit the cascade of inflammation [164]. Phosphorylation of ikB-α by IKK results in its degradation that ultimately causes activation and translocation of NF-kB into the nucleus [164]. NF-kB in the nucleus carry out expression of pro-inflammatory cytokines (Fig. 6). Literature supports the fact that NF-kB is one of the primary targets for cardiotoxic agents. Additionally, NF-kB plays a pivotal role in isoproterenol and ischemia-reperfusion (I/R) injury model. It has been previously demonstrated that NF-kB is associated with oxidative stress and inflammatory cascade in cardiac hypertrophy and cardiac fibrosis [51].

It is evident that upon exposure to cyclophosphamide, there is activation of NF-kB [6,126,164,165]. It is documented that phosphorylation of tissue growth factor-beta (TGF-β) activates NF-kB under the influence of IL-12 and TGF-β activated kinase [166]. Apart from NF-kB, experimental studies reported the elevation of tissue necrosis factor-alpha (TNF-α), IL-6, and IL-1 β followed by CP administration [126,164,165]. Recently Jiang et al., 2017, had shown that when cyclophosphamide was administered at the dose of 80 mg/kg i.p. on alternate days for three days and analyzed for inflammatory markers. CP treated group exhibited a marked elevation in TNF-α, p-NF-kB p65, p-IkBα, IL-6 and IL-1β [164]. Administration of magnesium isoglycyrrhizinate at the dose of 25 and 50 mg/kg, i.p. significantly mitigated these changes [164]. In another study, cyclophosphamide when administered at the dose of 200 mg/kg, i.p. resulted in the induction of creatinine kinase, IL-6, IL-1β, TNF-α, and NF-kB signaling pathways [6]. Treatment with ferulic acid at the dose of 50 and 100 mg/kg, i.p. for seven consecutive days sufficiently attenuated these inflammatory changes [6]. While discussing the cardiac inflammation and inflammatory cytokines, it must be kept in mind that these are not specific markers rather LDH, Troponin T and CK-MB are specific markers for cardiac injury [122,126,167]. Cyclophosphamide at the dose of 200 mg/kg, i.p. in a different set of experiments resulted in the elevation of CK-MB and Troponin T along with the inflammatory cytokines [126,167]. Omelo et al. 2017, conducted a pre-treatment regimen of 14 days experiment. CP was administered on the last three consecutive days at a dose of 50 mg/kg, i.p. After 24 h of the last dosing, animals were sacrificed. Cardiac-specific markers like LDH, CK-MB, and TnT were estimated using ELISA. cTnT, CKMB, and LDH were found to be significantly higher in the toxic group. Cardiotoxicity was further confirmed by histological findings [7].

4.8. Cyclophosphamide and apoptosis

It is a generalized idea that oxidative stress initiates the cascade of apoptosis [168]. Myocardial infarction, reperfused heart, diabetic-induced cardiomyopathy and left ventricular dysfunction often leads to apoptosis along with necrosis [169–172]. Experimental data supports that ROS activates the pro-apoptotic proteins and cause the release of cytochrome *c* via voltage-gated anion channels. In a healthy or normal

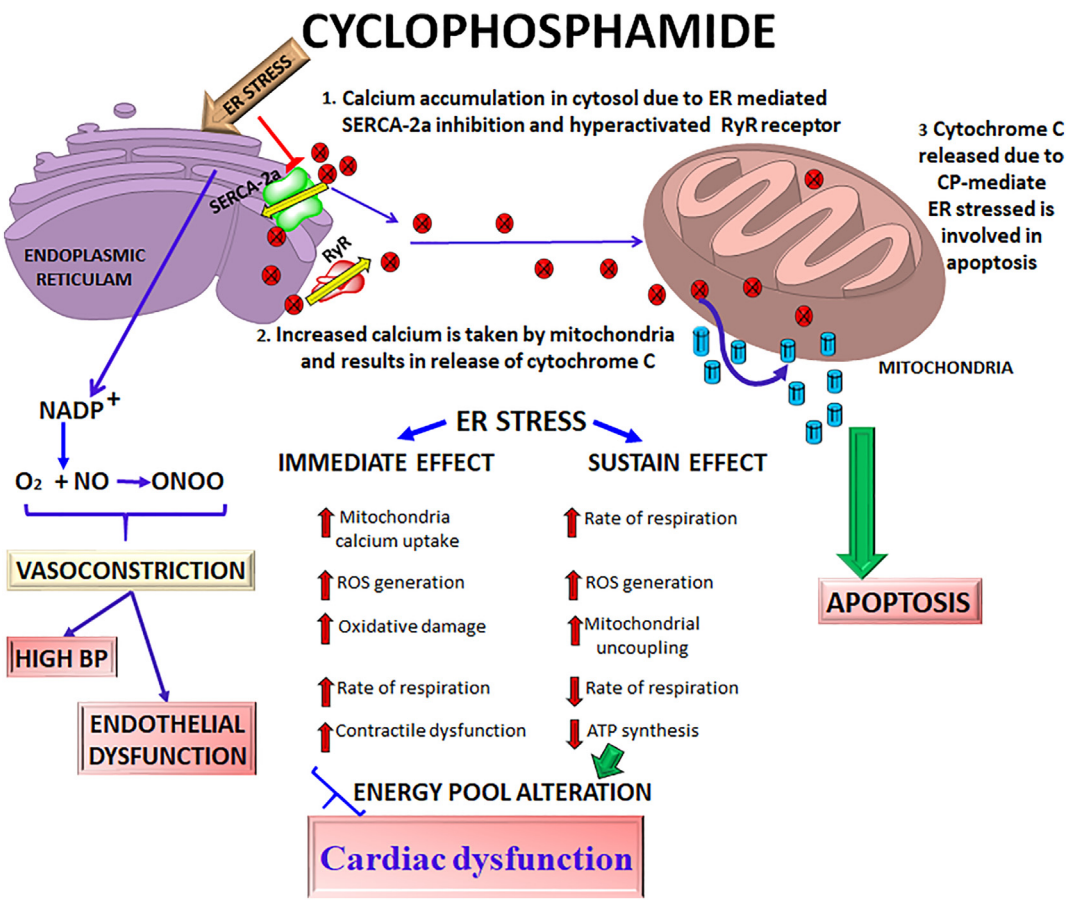

Fig. 4. Schematic diagram showing the effect of cyclophosphamide (CP) on the endoplasmic reticulum. 1. Calcium accumulation in cytosol due to ER-mediated SERCA2a inhibition and hyperactivated RyR receptor; 2. Increased calcium is taken by mitochondria and results in the release of cytochrome C; 3. Cytochrome C released due to CP mediated ER stress is involved in apoptosis; 4. $NADP^+$ induced nitrative stress causes vasoconstriction which leads to high blood pressure and endothelial dysfunction. These events collectively cause cardiac dysfunction.

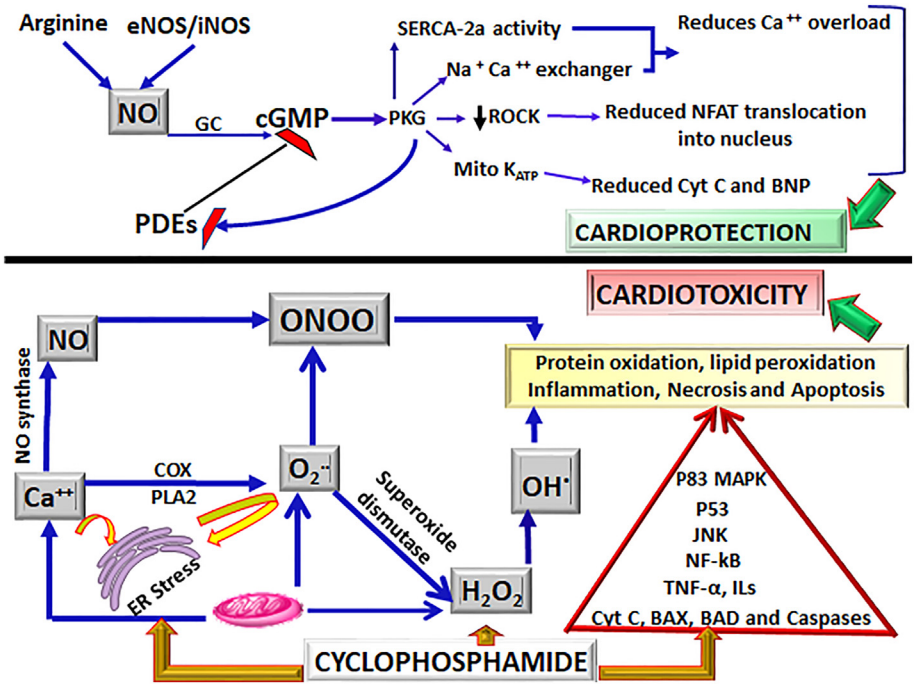

Fig. 5. Schematic diagram showing the effects of cyclophosphamide (CP) on nitric oxide. Thus, we can summarize that CP is responsible for mitochondrial insult, endoplasmic reticulum stress and increases in the level of oxygen free radicals. ER stress causes elevated calcium level which enhances the production of nitric oxide (NO). NO combines with ROS and form peroxynitrite radical ($ONOO^-$) which$^-$ further get involved in inflammation, apoptosis, necrosis and ultimately cardiotoxicity. In contrast to this, NO also exhibit cardioprotective role under physiological condition by increasing the level of cyclic guanine monophosphate (cGMP) and protein kinase G (PKG) via the action of guanine cyclase (sGC) enzyme. Increased PKG is responsible for cardioprotection by sequestering calcium into ER via stimulating sarcoplasmic reticulum calcium ATPase pump (SERCA-2a) and Na^+/Ca^{++} pump. PKG impede the effect of Rho activated protein kinase (ROCK), inhibits the action of phosphodiesterase (PDEs) and stimulates the activity of mitochondrial potassium ATP pump (Mito K^+ATP). Thus, overall effect is accountable for cardioprotection.

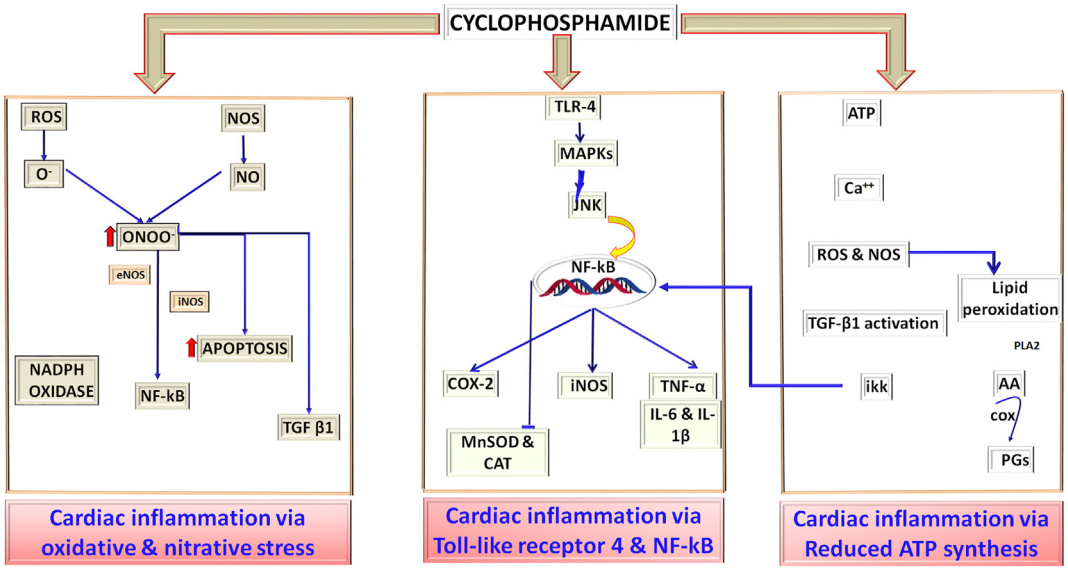

Fig. 6. Schematic diagram showing the initiation of cardiac inflammatory reaction by administration of cyclophosphamide CP via different pathways. CP induces oxygen and nitrogen reactive species (ROS and RNS) through increased oxygen free radicals and inducible/endothelial nitrogen synthase (iNOS and eNOS). Cyclophosphamide activates the toll-like receptor r (TLR-4) which in turn causes subsequent activation of mitogen-activated protein kinase & c-Jun N-terminal kinases (MAPK and JNK). These activated signaling pathways increase the expression of tissue necrosis factor alpha (TNF-α), cyclooxygenase-2 (cox-2), prostaglandins (PGs) and interleukins (ILs). Cyclophosphamide also diminishes the mitochondrial ATP production. Reduced ATP level results in the accumulation of intracellular calcium level which activate tissue growth factor beta (TGF-β) TGF-β phosphorylate IκB kinase (ikk) which in turn, phosphorylate IκBα, an inhibitor protein. This phosphorylation process results in dissociation of IκBα from nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB). Further, NF-κB translocates into the nucleus and get involved in the production of pro-inflammatory cytokines.

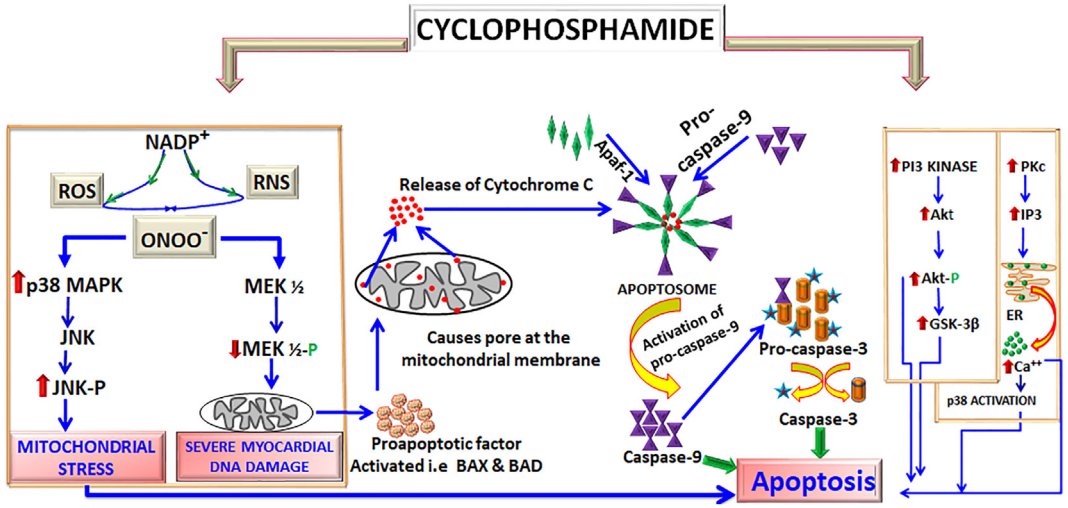

Fig. 7. Schematic diagram showing the proapoptotic role of cyclophosphamide (CP). CP increases the level of peroxynitrite which leads to activation of p38 mitogen-activated protein kinase and c-Jun N-terminal kinases (MAPK and JNK). Cyclophosphamide also diminishes the activity of mitogen-activated protein kinase-kinase (MEK1/2). Both the processes cause mitochondrial and cardiomyocyte damage. Mitochondrial damage results in the activation of pro-apoptotic factorsof Bcl-2 family protein like Bcl-2 associated death promoter (BAX and BAD) and subsequent release of cytochrome C. Cytochrome C induces the formation of apoptosome via apoptotic activated protease factor-1 (Apaf-1). Apaf-1 then activates procaspase-9 and caspase 3. Cyclophosphamide elevates the level of protein kinase B and glycogen synthase kinase-3 beta (Akt& GSK-3β). CP causes hyperactivity of protein kinase C and inositol triphosphate (PKC and IP3) that further increases the intracellular calcium level.

cell, mitochondria contain antiapoptotic genes like Bcl-2 [173] which on cardiotoxicity get inhibited by the proapoptotic proteins like Bax or BAD that migrate to the mitochondria. Pro-apoptotic proteins also cause holes in the outer mitochondrial membrane which offer a way for the escape of cytochrome *c*. Cytochrome c then combines with the apoptotic protease activating factor-1 (Apaf-1) in the presence of ATP. The complex so formed is called apoptosome [174]. Apoptosome binds with the procaspase-9 and activates it to form caspase-9. This activated caspase then triggers the activation of subsequent caspases like caspase 3, 7 and 12. This event as a whole is responsible for apoptosis (Fig. 7) [175]. Cyclophosphamide when administered in murine models as i.p. injection caused an increment in pro-apoptotic proteins and reduction in anti-apoptotic proteins [126]. Signal transduction pathways involved in cyclophosphamide-induced apoptosis and cardiac damage are PI3K/Akt/mTOR/p70S6K/4EBP1/NF-κB pathway, NF-kB/Nrf2-HO pathway, Akt/GSK3-β pathway, ERK1/2, p38 MAPK, JNK pathway and TLR4/NF-κB pathway [6,112,126,134,176,177]. Cyclophosphamide-induced apoptosis has been reported to be accompanied with cardiac inflammation and oxidative stress [128]. Clinical study also suggested the occurrence of cyclophosphamide-induced cardiac apoptosis which leads

to interstitial hemorrhage, cardiomyocytes macrovascular pathological alteration and cardiomyopathy [178].

4.9. Cyclophosphamide and p53 expression

One of the most commonly studied proteins in cancer as well as in cardiovascular research is p53 [179,180]. p53 primarily act as a transcription factor and modulate the pathogenesis of apoptosis by increasing the expression of the pro-apoptotic gene and reducing the expression of anti-apoptotic genes [181]. A recent study has shown the association between p53 expression and heart failure. Publications claimed the upregulation of p53 expression in ischemia, oxidative stress, tachycardia and mitochondrial stress caused by anthracyclines and CP [181–183]. Studies have shown that inhibition of p53 exhibits cardioprotection [184]. More precisely, p53 inhibition results in reduced apoptosis, reduced infarct size and cause improvement in hemodynamic parameters [180]. People have pointed out the role of p53 in cardiotoxicity by using p53 knockout mice where they have shown a reduction in the extent of toxicity induced by anticancer drugs [185].

4.10. Cyclophosphamide and p38 MAPK expression

p38 MAPK (mitogen-activated protein kinase) is the class of MAPKs accountable for inflammation and apoptosis [95]. p38 MAPKs is reported to be activated by a various stimulus, like drugs (doxorubicin, cyclophosphamide, isoproterenol, arsenic trioxide, etc.), pressure overload, I/R, oxidative stress, sepsis, UV light and lipopolysaccharides (LPS) [185]. p38 MAPKs are activated by phosphorylation at Thr180 and Tyr182 residues [185,186]. Activated p38 then activate ATF-2 (Activating transcription factor 2) which is responsible for the expression of c-Jun N-terminal kinases (JNKs) [187]. In cardiology p38 activation is considered to be a mediator of cardiac damage [188]. Bao et al., 2007, reported that angiotensin-II (Ag-II) induced p38 activation causes hypertension which was reversed by using SB239067, a p38 inhibitor [189]. Role of p38 was further strengthened by the findings of Koivisto et al., 2011, who reported an increase in the expression of mRNA of hypertrophic genes like BNP and ANP [190]. The same inhibitor was used by other researchers to prove its cardioprotective potential [188,191–193].

From the above-discussed studies, it seems that phosphorylated p38 MAP Kinase is responsible for arrhythmias, tachycardia, MI, Left Ventricular remodeling, cardiomyopathy, and heart failure but this is not always being the case. Tanhunen et al., 2006, had reported that p38 activation is responsible for reduced infarct size, enhanced capillary density, improved ejection fraction, reduced apoptosis and fibrosis [194]. The possible explanation for this contrast result could be the crosstalk between p38 MAP kinase and GSK-3β. As it is established that p38 MAPK is responsible for the phosphorylation of GSK-3β, the phosphorylated GSK-3β results in the inhibition of apoptosis by inactivating BAX & p53 [194–196]. Overall, it has been concluded that p38 MAPK is accountable for cardiotoxic effects [193]. Seeing the cardiotoxic effect of p38, p38 inhibitors like losmapimod (GSK), ARRY-371797 (Array BioPharma) and BMS-582949 (Bristol Mayer Squibb) have been developed [119,120]. Losmapimod has passed phase 1 and phase II clinical trials which established its safety and efficacy regarding maintaining ejection fraction and left ventricular end-diastolic and end-systolic volumes. Based on the result of phase I & II, phase III trial has been initiated with the name “LATITUDE-TIMI 60” (Losmapimod to inhibit p38 MAPK as a therapeutic target and modify outcomes after an acute coronary syndrome-thrombolysis in myocardial infarction 60; NCT02145468). This trial has incorporated 26,000 patients and drug was given at the dose of 7.5 mg, BID for 12 weeks. The result of this trial is likely to be public at the at the end of 2018 [95]. CP when given in animal models also resulted in the elevation of p38 and another signal transducer of MAPKs alongwith other mediators of inflammation and apoptosis responsible for its cardiotoxic effect [126,128].

4.11. Cyclophosphamide and GSK-3β, Akt/PI3K/signaling

GSK-3β is a Ser/Thr protein kinase. In human GSK-3β is encoded by GSK-3B gene whereas in mice it is encoded by GSK-3β gene [197]. GSK-3β negatively regulates its downstream signaling cascade and get deactivated on phosphorylation by stimuli like pressure overload, hypertrophic stimuli, Wnt signaling, Akt/PI3K pathways, protein kinase A and protein kinase C [197–199]. Once GSK-3β is inactivated/phosphorylated, it carries out diverse cellular functions like hypertrophy and heart failure [199,200]. Phosphorylation site for GSK-3β has been identified as Ser21 for GSK-3α and Ser9 for GSK-3β [197]. As we have mentioned earlier, Akt/PI3K is responsible for GSK-3β inactivation/phosphorylation, so it is understood that stimuli activating Akt/PI3K will also inactivate/phosphorylate GSK-3β and will participate in cardiac hypertrophy and heart failure [201]. Stimulus like endothelin-1 also phosphorylates GSK-3β and thus is involved in cardiac hypertrophy [202]. Association between phosphorylated GSK-3β and cardiac hypertrophy was further proved by the experiment where the mutant form of GSK-3β was used. Mutant GSK-3β did not allow phosphorylation at Ser9 and thus participated in inhibiting hypertrophy in an isoproterenol-inducedmodel [199,201–204]. Mechanism of cardiac hypertrophy involves the role of transcription factor-like NFAT, translational factors like eF2B, 4E-BP, eEF2 and cytoskeleton structures like actin [205]. Protein synthesis is crucial for hypertrophy. One of the critical steps in protein synthesis is the association of eIF2 with activated t-RNA (met-tRNA). This complex then binds to the 40s subunit of the ribosome [205]. eIF2B is another elongation factor required for the exchange of GDP/GTP reaction of eIF2 [205]. In the normal or unstimulated cell, where GSK-3β is unphosphorylated/activated, causes phosphorylation of eIF2ε and thus makes it inactive [199,201,204]. Upon phosphorylation/deactivation, GSK-3β cannot phosphorylate eIF2ε and thus eIF2ε carries out the process of protein synthesis, and ultimately hypertrophic heart is formed [199]. Therefore, it becomes clear that phosphorylated/deactivated GSK-3β at Ser9 contribute to cardiac hypertrophy (Fig. 9).

4.12. Cyclophosphamide, GSK-3β and NFAT

In the previous section, we have discussed the mechanism of hypertrophy mediated by deactivation/phosphorylation of GSK-3β under the influence of various stimuli. NFAT (nuclear factor of activated T-cell) is a positive regulator of cardiac hypertrophy [206–208]. NFAT belongs to the family of calcium-regulated transcription factors [206,209]. NFAT has an N-terminal regulatory domain that is responsible for its translocation to the nucleus [206,209]. In normal condition/phosphorylated state, NFAT remains sequestered in the cytoplasm [206,210]. Calcineurin which is activated under the influence of Ca^{++} and calmodulin protein, dephosphorylate NFAT at its regulatory domain [206]. Dephosphorylation of NFAT causes a conformational change that enables it to translocate into the nucleus. Once NFAT is translocated into the nucleus, it carries out the transcriptions of various genes for cardiac hypertrophy [206–208]. GSK-3β when remains unphosphorylated/active, causes phosphorylation of NFAT and restricts its entry into the nucleus and thus prevents cardiac hypertrophy [206–211]. Conversely, phosphorylated GSK-3β gets devoid of the ability to avert NFAT translocation and thus participates in cardiac hypertrophy [211] (Fig. 8). Interestingly, agents responsible for dephosphorylation/activation of GSK-3β may act as an anti-hypertrophic agent. One such example is Lithium. Lithium a metallic ion inhibitor of GSK-3β phosphorylation [212,213]. On the other hand, PI3K/Akt/PkA, PKc/cAMP acts as pro-hypertrophic agents causing phosphorylation/deactivation of GSK-3β [213]. In brief, GSK-3β as anti-hypertrophic refers to its unphosphorylated/active form whereas pro-hypertrophic GSK-3β refers to its phosphorylated/inactivate form.

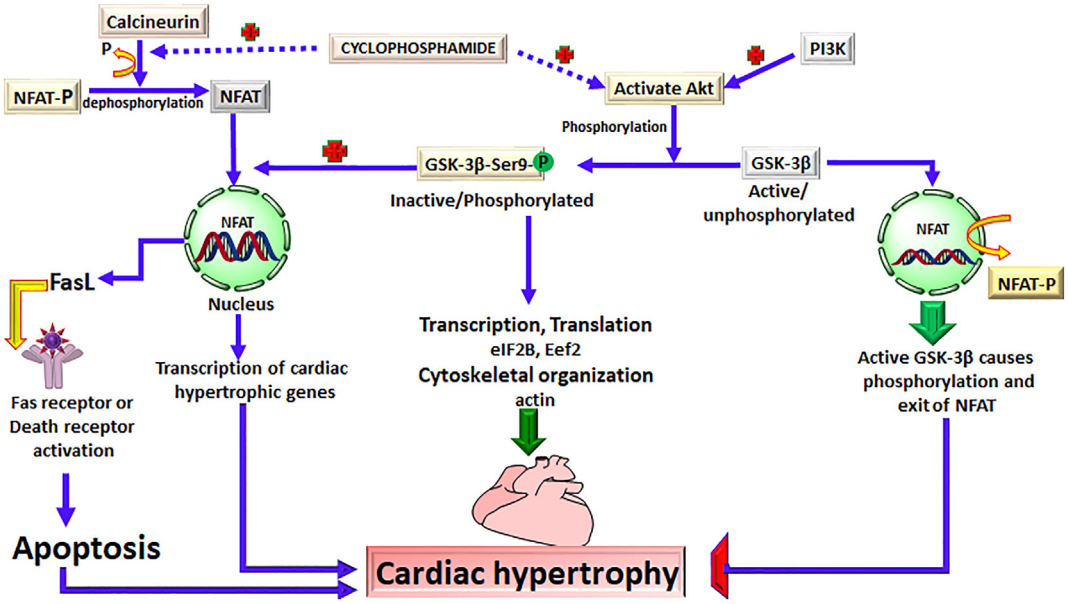

Fig. 8. Showing the effect of cyclophosphamide (CP) on NFAT and GSK-3β. CP induces the calcineurin-mediated dephosphorylation of NFAT which enters into the nucleus where it carries out the transcription of hypertrophic genes. Nuclear translocated NFAT also causes apoptosis via FasL or death receptor. Calcineurin also causes activation of Akt that is involved in phosphorylation of GSK-3β at serine 9. Phosphorylated GSK-3β is unable to remove the translocated NFAT from the nucleus and thus favors the cardiac hypertrophy, moreover unphosphorylated GSK-3β causes phosphorylation and exit of NFAT from the nucleus and therefore prevents cardiac hypertrophy.

GSK-3β in its unphosphorylated/active state also phosphorylates c-jun [214]. Phosphorylated c-Jun can'tbindto DNA and is unable to interact with mitogen-activated protein kinase, and thus transcription cascade for cardiac hypertrophy is inhibited [87,215]. Phosphorylated/inactive GSK-3β, on the other hand, cannot phosphorylate c-junand therefore c-Jun binds with DNA and carry out the transcription cascade [216]. To summarize the above-discussed event, we can say that unphosphorylated/active GSK-3β phosphorylates eIF2, phosphorylates NFAT, phosphorylates c-jun and thus contributes significantly to cardiac hypertrophy inhibition.

While discussing the GSK-3β and NFAT pathway, it is worth mentioning the study of Ibrahim AAl-Nasser 1998, where he showed the cardioprotective role of cyclosporine A against cyclophosphamide-induced cardiotoxicity [217]. Cyclosporin A binds with cyclophilin and form complex which further binds with calcineurin and inactivate it (Fig. 8). Thus, the inactivated calcineurin is unable to translocate NFAT into the nucleus and NFAT mediated cardiotoxicity/cardiac hypertrophy is inhibited. Additionally, nuclear translocated NFAT is also reported to be involved in apoptosis by increasing the production of Fas Ligand (FasL) which binds with the Fas receptors located in the membrane and apoptotic cascade is initiated through caspase 3, 6, 7 and 8. Cyclosporin A is also reported to inhibit apoptosis via blocking this NFAT/Fas signaling pathway [218]. Thus, our discussion for the involvement of cyclophosphamide-induced cardiotoxicity via GSK-3β and NFAT pathway is in agreement with the previous findings [217].

4.13. Cyclophosphamide and calcium dysregulation

As we have discussed above, beside the different mechanism of cardiotoxicity like energy alteration, cardiac inflammation, cardiac apoptosis, generation of reactive oxygen species, calcium also plays a crucial role in cardiotoxicity. Discussion of calcium is always associated with the endoplasmic reticulum (ER) and mitochondria. Calcium being a secondary messenger plays a diverse role, ranging from neuromuscular to cardiac muscles contraction [219]. The appropriate level of calcium in sarcolemma (cell membrane) is maintained by L-type calcium channel (LTCC), smooth endoplasmic reticulum calcium-ATPase pump (SERCA2a), ryanodine receptor and inositol triphosphate receptor (RyR& IP3R) [219]. Excitation-contraction (EC) coupling manage the contraction of cardiomyocytes [220]. EC coupling causes an increase in cardiomyocytes cytosolic calcium concentration [220]. Being more specific, calcium is sequestered from ER via LTCC and RyR. During the systolic signal, there is depolarizations of the sarcolemma (cell membrane) which causes entry of small amount of Ca^{++} into ER via LTCC [221]. This extracellular calcium binds with RyR and results in the massive efflux of Ca^{++} from ER into sarcoplasm [116]. This phenomenon is termed as calcium-induced calcium release (CICR). CICR is reported to be responsible for ten times higher concentration of Ca^{++} in the sarcoplasm [221]. Calcium then binds to contractile protein like troponin and result in cardiac contraction. Relaxation or diastole following contraction requires calcium to be removed from the sarcoplasm. 30% of calcium is removed from sarcoplasm via LTCC, and 70% of calcium is pumped back into ER via SERCA-2a [116]. Any impairment in this calcium regulation results in a diverse range of cardiac toxicity (Fig. 9). Antineoplastic drug-induced calcium dysregulation is well documented and accountable for cardiomyopathy and heart-failure along with oxidative stress, apoptosis and endothelial dysfunction [115]. In the case of cyclophosphamide, to the best of our knowledge, there is no reported information which correlates calcium dysfunction with cardiotoxicity. Studies have shown the altered ATP generation and mitochondrial dysfunction upon cyclophosphamide administration. Therefore, we suggest for exploration of cyclophosphamide on calcium dysfunction involving mitochondria and endoplasmic reticulum stress. Studies can be conducted under the following subclasses (a) cytosolic calcium overload. (b) Reduced calcium uptake from the cytosol into ER, (c) ER Calcium leak and (d) Energy crisis.

5. Cyclophosphamide, natriuretic peptide, cardio fibroblasts and endothelial dysfunction

In CP associated cardiomyopathy, the role of brain natriuretic peptide (BNP) is of prime [46] importance. BNP is a cardiac neurohormone which is released by the ventricular myocardium upon pressure load of myocardial stress and heart failure [219]. To the best of our knowledge, there is one published paper that states about the elevation

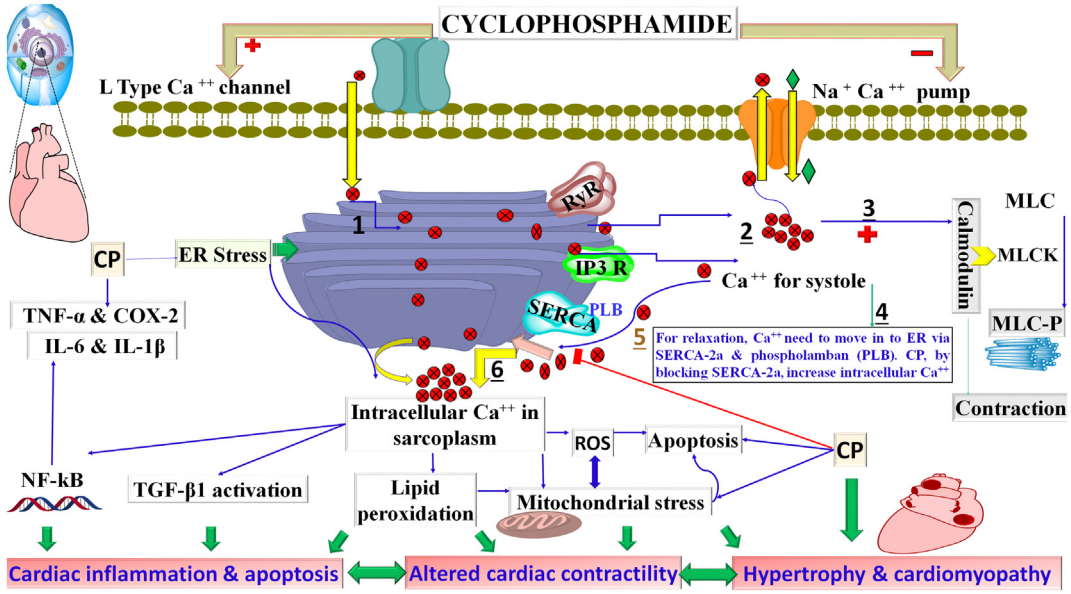

Fig. 9. Diagram showing the effect of cyclophosphamide (CP) on calcium dysregulation. Under normal condition, calcium is influxed into the endoplasmic reticulum (ER), through L type calcium channel. Calcium overload is the result of the pathological condition during CP mediated ER stress. During systolic stimulus, calcium is released from the ER into the cytoplasm via ryanodine receptor and inositol triphosphate receptor (RyR and IP3R). For relaxation, calcium must be sequestered into the ER. However, after administration of CP, impaired sequestration of calcium occurs in response to reduced/inhibited sarcoplasmic reticulum calcium ATPase pump activity or dephosphorylation of phospholamban (SERCA2a and PLB). Increased cytoplasmic calcium leads to the lipid peroxidation, mitochondrial stress, activated tissue growth factor beta, nuclear factor kappa-light-chain-enhancer of activates activated B-cells (TGF-β & NF-κB) and increased reactive oxygen species (ROS) level. ROS further inhibits SERCA-2a and phosphorylate PLB. The cumulative effect of calcium dysregulation is altered cardiac contractility and cardiac hypertrophy.

of BNP and ANP upon cyclophosphamide administration in rat model at the dose of 100 mg/kg, i.p. [128]. Clinical study has shown increased BNP level upon cyclophosphamide administration, which was positively correlated with cardiac dysfunction [222]. European guideline for the diagnosis of chronic heart failure also recommends the measurement of BNP as a biomarker of cardiac damage [223]. Apart from the involvement of BNP and ANP, cyclophosphamide-induced endothelial dysfunction and cardiofibroblasts is also well documented.

Myocardium consists of three different types of cell, cardiomyocytes, cardio fibroblasts (CFBs) and endothelial cells. In the previous section, we have discussed in detail about the potential cardiotoxic effect of CP on cardiomyocytes. This section deals with the cardiotoxic effect of CP on CFBs and endothelial cells. Fibroblasts (FBs) are mesenchymal connective tissue, present all over the body including cardiovascular system, where it is known as cardiofibroblasts (CFBs) [224]. In cardiovascular System, CFBs accounts for the two-third of cells. CFBs originate from mesenchymal cells via epithelial-mesenchymal transformation (EMT), and from endothelial cells (endocardium) via endothelial-mesenchymal (EndMT) transformation. [225]. Under normal condition, CFBs are responsible for maintaining cardiac homeostasis, communicate with cardiomyocytes via gap junction, and regulate contraction-relaxation [226]. CFBs are also involved in intracellular signaling, angiogenesis, maintenance of cardiac structural integrity and biochemical-electrical events [226,227]. CP or its metabolites cause activation and transformation of cardiofibroblast into myofibroblast (myoFBs). There is a growing evidence that CP induces the increased expression of TGF-β that further potentiate the transformation of FBs into myoFBs (Fig. 10) [228]. myoFBs are present only during pathological condition and are responsible for the production of inflammatory cytokines like IL-6, IL-1β, TNF-α, MMPs, and BNP [229]. Increased level of inflammatory mediators and myoFBs under the influence of CP results in cardiac inflammation and causes stiffness of the myocardium, altered ventricular contraction, fibrosis and cardiomyopathy [230]. CP-induced activation of CFBs and transformed myoFBs also interrupt the myocytes-myocytes interaction, involved in macrophage recruitment and bone marrow alteration. Altered bone marrow further leads to the activation and migration of neutrophils, monocytes cyclooxygenase-2, lipoxygenase, 12/5 HETE, and leukotriene-4 to cardiac tissue. The cumulative effect of activated cardiofibriblastleads to cardiac inflammation and impairment in the cardiac function (Fig. 10) [229,231]. Effect of CP or its metabolite on fibroblast is further evident from work done by Kamaya et al., 1998, where leukotriene B4 (LTB4) antagonist and Monocyte Chemoattractant Protein-1 (MCP-1) antibodies augmented the CP-induced activation of fibroblast and subsequent chemotactic activity [232]. Therefore, it can be concluded that under the pathological condition of CP-induced TGF-β production, cardio fibroblast transform into myofibroblast. Myofibroblast undergo inflammatory phase where myoFBs increases the level of TNF-α, IL-6, IL-1β & MMPs. In the proliferative phase, myoFBs undergoes proliferation and fibrosis whereas during the maturation phase, myoFBs undergoes apoptosis. The overall effect progress into myocardial infarction, cardiac fibrosis, cardiac hypertrophy, cardiomyopathy, and heart failure.

Vascular endothelium is important active autocrine, endocrine and paracrine part of cardiovascular system that play a vital role in the maintenance of homeostasis and vascular tone [233–235]. In general, endothelium refers to cells that line the inner surface of blood vessels and form an interface between circulating blood in the lumen and the rest of the vessel wall. Endothelium is a thin layer of simple, or single-layered, squamous cells called endothelial cells. Endothelial cells that are in direct contact with blood are called vascular endothelial cells [233].

Under normal physiological condition, the endothelium is responsible for the regulation of cellular adhesion, thromboresistance (thrombosis and thrombolysis), vascular inflammation and maintenance of the vascular contractive property [233,236]. Of particular interest, endothelium sense the hemodynamical change and accordingly respond to the stimulus by the release of vasoactive substance like vasorelaxants (NO, prostacyclin and EDRF), vasoconstrictor (endothelin-1) and antithrombotics (plasminogen activators) [236]. As

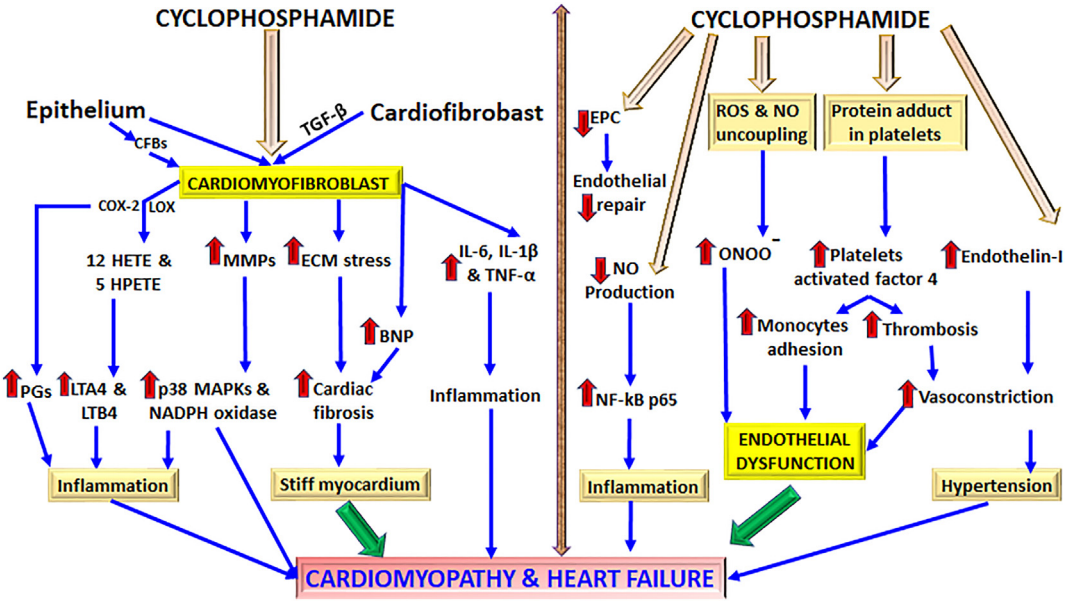

Fig. 10. Showing the toxic effect of cyclophosphamide (CP) on cardiofibroblast and endothelial cells. CP causes transformation of cardiofibroblast (CFBs) to myofibroblast (myoFBs) which is a pathological state of CFBs. MyoFBs imitate the oxidative and inflammatory response in cardiac cells by activation and migration of NADPH oxidase, ILs, TNF-α, prostaglandins and leukotrienes (LTA4 and LTB4.MyoFBs also causes cardiac fibrosis and heart failure under the influence of (Brain Natriuretic Peptide) BNP and MMPs. Additionally, CP causes endothelial dysfunction (ED) by reducing nitric oxide production and endothelial progenitor cells. CP-induced ED is also mediated by increased activity of endothelin-1, NF-kB, monocyte adhesion and platelet activated factor 4, that together leads to vasoconstriction and hypertension.

shown in Fig. 5, NO produced from L-arginine under the expression of eNOS diffuses into vascular smooth muscles and regulate the vasorelaxant function. Apart from the vasorelaxant effect, NO (eNOS) also act as an anticoagulant, antiproliferative, antithrombotic and anti-inflammatory molecule. Anti-inflammatory effect of NO is mediated by its interaction with cysteine that modulates and keeps the NF-kB (p50/p65) at inactivated state.

CP or its metabolite causes direct damage to the endothelial cells and alter its physiological function [24]. CP has been reported to induce oxidative stress that causes an increase in the permeability of vascular endothelium & initiate the cascade of leucocytes adhesion leading to alteration in endothelial signal transduction and endothelial dysfunction (ED). CP or its toxic metabolite form protein adduct in the platelets and causes activation and release of platelets activated factor 4 (PF-4), which induces the cascade of thrombosis [237]. Toxic metabolites of CP also initiate the binding of oxidized LDL to endothelial cells and aggravate the monocyte adhesion to endothelium leading to endothelial dysfunction and vasoconstriction [237]. Endothelial progenitor cell (EPC) is an important part of endothelium involved in repair and angiogenesis. There are also reports of CP or its metabolite mediated reduction in endothelial progenitor cell (EPC) via depression of bone marrow that reduces the ability to repair the damaged cell and causes subsequent ED [238]. CP or its metabolite also causes inhibition of eNOS phosphorylation leading to reduce NO production, reduced eNOS dimer and increased eNOS monomer formation that causes CP-induced eNOS uncoupling. eNOS uncoupling progress into the formation of peroxynitritethat has a diverse pathological profile in the cardiovascular system (Fig. 10) [27]. Clinically, there are growing evidences for alteration in endothelin-1 (ET-1) upon CP administration [40]. ET-1 is an important vasoactive substance released from endothelium in order to counter balance the effect of NO [239]. ET-1 acts on the ET_A and ET_B receptors located all over the cardiomyocytes, coronary vessels and endothelial cell [239]. Overproduction of ET-1 under the cardiotoxic influence of CP causes a reduction in cardiac output, and increases cardiac overload [240]. As discussed in the previous section, during alteration in LVDF and cardiomyopathy, a commonly oberved CP-induced cardiotoxicity, consistent and profound increase in the level of ET-1 have been reported [100,128]. Recently, ET-1 induced activation of MMPs, mast cell, and hypertrophic myocardial remodeling have shown induction of IP3/PKC, that lead to increases in intracellular Ca^{++} concentration, resulting in hypercontractility, inflammation, and apoptosis in blood vessels, cardiofibroblasts, and cardiomyocytes [241]. There are also reports for the down-regulation of eNOS mRNA, reduction in the level of tetrahydrobiopterin (cofactor needed for NO synthesis), increase in eNOS uncoupling, oxidative stress and generation of peroxynitrite under the influence of ET-1 [242]. Apart from the effect on vasomotor function, CP-induced ET-1alteration has been reported to activate and increase the chemotactic role of TNF-α, macrophages, monocyte adhesion to the blood vessels and endothelium [241]. It also activates NF-kB pathway leading to endothelial inflammation and endothelial dysfunction (Fig. 10) [243].

CP hasalso been reported to cause extravasation of proteins, toxic metabolites and erythrocytes which causes breakdown of endothelial cells, obstructs the small arteries and causes dislodgement of vascular endothelial cells that directly damages the cardiac cells, blood vessels and cause interstitial hemorrhage [19]. CP or its metabolites also causes intramyocardial extravasation of fibrin or fibrin-platelet microthrombi in the blood capillary and interstitium [97]. Capillary thrombosis and fibrin deposition in the myocardial interstitial are unique mechanisms of CP-induced cardiotoxicity [46].

Therefore, it can be concluded that CP or its metabolite causes an alteration in vasoactive substance like reduction in NO, increase in ET-1 and in the activity of plasminogen activating factor leading to oxidative and nitrative stress, aggravate endothelial inflammation and thrombosis. These attributes are responsible for hypercontraction, vasospasm, mitochondrial dysfunction, hypertension, coronary artery disease, and heart failure.

6. Discussion

Cyclophosphamide is widely used as an anticancer drug and routinely used as a regimen for hemopoietic stem cell transplant. Use of CP came into existence after the second world war, and soon it was realized to be associated with dose-dependent cardiotoxicity [9,12]. The first

case of CP-induced cardiotoxicity was reported by Santels et al., 1971, soon after, several reports of CP-induced cardiotoxicity were published [17–19,38–50]. For the last few decades people have been fully focussing on understanding the pharmacokinetics of CP. It is stated that CP is a prodrug and requires cytochrome P450 isozymes for its bioactivation in the presence of cytochrome P450 isozymes [7,12–17]. Bioactivation results in the formation of 4-HCYP and aldophosphamide which undergo β elimination to form phosphoramide mustard and acrolein [13,17]. Acrolein is an α-β unsaturated aldehyde and found primarily to be cardiotoxic [244]. Previous studies have suggested that not only acrolein but 4-HCYP is also associated with cardiotoxicity [12].

Cyclophosphamide and their metabolites are reported to increase the susceptibility of myocardium, endothelium and cradiofibroblasts towards necrosis and myocardial depression [245]. Cyclophosphamide and their metabolites also form adducts with cardiomyocytes α-actin, cystiene, desmin, platelet activating factor-4 and myosin light chain kinase polypeptides leading to subsequent cardiotoxicity [25]. Studies have reported that the activation of MMP-9 disrupts the atherosclerotic plaque and causes coronary occlusion [245–247]. Myeloperoxidase is another key regulator responsible for alteration in left-ventricular contraction and cardiac remodeling [25].

Till date, themolecular mechanism of CP-induced cardiotoxicity is not fully understood. However, based on previous studies, it has been postulated that oxidative stress may play a crucial role in CP-induced myocardial damage [7,123–128]. Few studies have reported H-FABP alteration and endothelial damage in cyclophosphamide-induced cardiotoxicity model [248]. H-FABP is a protein responsible for the transportation of long chain fatty acid from the myocardial cytosol to myocardial mitochondria [107].

It is important to highlight that in cardiac muscles, fatty acid oxidation is a primary source of ATP production via fatty acid oxidation (β-oxidation) [104]. Any alteration in H-FABP directly results in impairment of ATP production leading to myocardial damage via oxidative stress, apoptosis and inflammation [105,111]. CPT-1, ACCb, MCD, and malonyl CoA are a key regulator of β oxidation. CPT-1 is responsible for the transportation of long chain fatty acyl CoA into the mitochondrial matrix [104,105]. CPT-1 is allosterically inhibited by the Malonyl CoA which is synthesized from acetyl CoA via ACCb (carboxylation) and degraded by the MCD (decarboxylation) (Fig. 2). As discussed earlier CP administration at the therapeutic dose stimulate the activity of malonyl CoA, acetyl CoA, and ACCb, whereas it downregulates the activity of MCD and thus participates in cardiotoxicity [105].

It is important to discuss here that although β oxidation is essential for the normal working of the heart, excess fatty acid oxidation leads to contractile dysfunction and dilated cardiomyopathy because fatty acid oxidations consume more ATP than glucose metabolism in terms of per oxygen molecule consumed [104–109]. Upon complete oxidation of a molecule of glucose, six molecules of oxygen are consumed, and 31 ATP is produced whereas complete oxidation of palmitate yield 105 ATP and consume 23 molecules of oxygen [104]. This difference in P/O ratio is accountable for 10% reduction in cardiac efficiency. Apart from reduced P/O ratio between β oxidation and glucose oxidation, mitochondrial uncoupling, futile cycling and increased level of intracellular calcium from sarcolemma calcium channel contribute to reduced cardiac efficiency [104]. Studies also reported that increased fatty acid oxidation leads to the impaired translocation of ATP from the mitochondrial matrix to the cytosol for efficient contraction [249]. Randle cycle or the glucose/fatty acid cycle plays a crucial role in maintaining the balance between glucose metabolism and β oxidation and balance the P/O ratio in the physiological heart [250]. Therefore, based on the literature, we conclude that constant high demand of energy to sustain the contractile action of heart is supplied by β oxidation whereas in pathological conditions like diabetes, obesity, and ischemia-reperfusion excessive, β oxidation results in the reduction of cardiac efficiency by 30% and contribute to heart disease [104].

During the late 19th century it was reported that CP at high dose results in endothelial damage along with extravasation of plasma protein. Several studies have shown the leakage of CPK, LDH, AST & ALT during CP-induced cardiotoxicity [7,123–128]. CP administration has been reported to activate myocardial xanthine oxidase that further catalyzes the formation of hypoxanthine to xanthine. Xanthine then forms hydrogen peroxide and uric acid. Increased uric acid is responsible for kidney damage whereas hydrogen peroxide results in oxidative stress and may react with NO causing nitrative stress. No doubt, these enzymes are not the direct marker of cardiac injury, but they do give an idea of the cardiac damage. Inflammation is a known progenitor of cardiac damage and several studies have shown the elevation of pro-inflammatory cytokines like IL-6, IL-10,and IL-1β during cardiac damage. Oxidative stress is the main culprit behind the pathogenesis of cardiac inflammation. In fact, the inflammatory process is a complex phenomenon which involves multiple signaling pathways. TLR-4 is an essential activator of proinflammatory cytokines and results in the activation of MAPKs and NF-kB. Study have also shown the elevation of TLR-4 followed by single i.p. injection at the dose of 200 mg/kg [251]. NF-kB is a well-known mediator of inflammation and once NF-kB is activated, it induces the expression of TNF-α, NO and other proinflammatory mediators. Several studies have shown that amelioration of the NF-kB pathway results in cardiac protection [6,126]. Thus TLR-4/NF-kB/MAPKs pathways are essential cascade in CP-induced cardiotoxicity.

While discussing the inflammatory role of NF-kB, it is important to highlight that NF-kB is closely associated with apoptosis because it regulates Bcl-2 transcription [128]. Apoptosis has been identified as a marked indicator of cardiac injury along with oxidative stress/nitrative stress and inflammation [174]. Apoptosis is marked by the changes in pro-apoptotic genes like Bax and antiapoptotic genes like Bcl-2 and Bcl-xL [95]. As mentioned earlier, increased oxidative stress and inflammation results in the translocation of Bax to the outer mitochondrial membrane where it causes an alteration in mitochondrial permeability. Increased permeability induces the release of cytochrome C into the cytosol. This process carries out the formation of apoptosome and other downstream effectors like caspases [155–164]. On the other hand, Bcl-2 helps in the preservation of mitochondrial wall integrity and inhibits the release of cytochrome C [160–161]. Recently Liu et al., 2015, have shown the increased expression of pro-apoptotic genes in the cardiac tissues and reduced expression of Bcl-2 (anti-apoptotic) genes after CP treatment [128]. The author also suggested that the activation of NF-kB and apoptosis were closely relatedto CP administration. Although studies have demonstrated the role of TNF-α, NF-kB, TLR-4, MAPKs, p53, Nrf-HO, diminished ATP production, mitochondrial stress and altered H-FABP in CP-induced cardiotoxicity. We suggest an alternate pathway for cardiac injury, i.e., ER stress mediated by calcium dysfunction. ER stress is well established in anthracycline, isoproterenol and I/R injurymodels along with ROS, inflammation, apoptosis and energy alteration. Thus, there is a highest probability that CP may play a prominent role via ER-mediated cardiac injury. ER stress comprises increased calcium concentration in the cytosol, alteration in RyR, IP3-R and phospholamban activity. Further ER-induced increased calcium in sarcoplasm is manifested by apoptosis, lipid peroxidation and mitochondrial stress resulting in altered contraction-relaxation leading finally to hypertrophy and heart failure.

7. Conclusion

Based on our proposed mechanistic pathways derived from published evidences, we conclude that CP is associated with dose-dependent cardiotoxicity mainly myocardial microvascular pathological changes, myocytes necrosis, and interstitial hemorrhage. Cardiotoxicity by CP is not only the result of oxidative stress as previously reported in many studies, but multiple pathways are involved in this toxicity. Myocardial inflammation, apoptosis, endothelial damage and alteration

in H-FABP and compromised ATP production are primarily responsible for the cardiac injury. Co-administration of agents that can ameliorate oxidative stress, nitrative stress, uphold H-FABP and preserve endothelial integrity can be of clinical significance in the mitigation CP-induced cardiotoxicity.

Conflict of interest

None.

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