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Doxorubicin-Induced Oxidative Injury of Cardiomyocytes – Do We Have Right Strategies for Prevention?

Vukosava Milic Torres¹ and Viktorija Dragojevic Simic²

*¹Laboratory of Proteomics, Department of Genetics,
National Institute of Health Dr Ricardo Jorge, Lisbon,*

*²Center for Clinical Pharmacology,
Military Medical Academy, Belgrade,*

¹Portugal

²Serbia

1. Introduction

Anthracyclines are among the most utilised antitumour drugs ever developed. The discovery of one of the leading compounds, doxorubicin (DOX) in early 1960s was a major advance in the fight against cancer. According to the WHO, it belongs to the group of 17 essential drugs that are used to treat curable cancers or cancers for which the cost-benefit ratio clearly favours drug treatment (Sikora et al., 1999). It is used, often with other antineoplastic, in the treatment of Hodgkin's disease, non-Hodgkin's lymphomas, acute leukaemias, bone and soft-tissue sarcoma, neuroblastoma, Wilm's tumour, and malignant neoplasms of the bladder, breast, lung, ovary, and stomach. The mechanisms of cytotoxicity of DOX in cancer cells is complex including: inhibition of both DNA replication and RNA transcription; free radicals generation, leading to DNA damage or lipid peroxidation; DNA cross-linking; DNA alkylation; direct membrane damage due to lipid oxidation and inhibition of topoisomerase II (Gewirtz, 1999; Minotti et al., 2004). Today, topoisomerase II is generally recognized to be the cellular target of DOX, which act by stabilizing a reaction intermediate in which DNA strands are cut and covalently linked to this enzyme (Simunek et al., 2009). It blocks subsequent DNA resealing. Failure to relax the supercoiled DNA blocks DNA replication and transcription. Furthermore, DNA strand breaks may trigger apoptosis of cancer cells. However, as with all traditional antineoplastic drugs, DOX administration is accompanied by adverse drug reactions arising from the limited selectivity of their anticancer action (Aronson et al., 2006; McEvoy et al., 2010). Particularly common are bone marrow depression, which may be dose-limiting. White cell count reaches a nadir 10 to 15 days after a dose and usually recovers by about 21 days. Gastrointestinal disturbances include moderate or sometimes severe nausea and vomiting; stomatitis and oesophagitis may progress to ulceration. Alopecia occurs in the majority of patients. Occasional hypersensitivity reactions may also occur. However, a cumulative-dose dependent cardiac toxicity has been a major limitation of DOX use.

2. Doxorubicin-induced cardiotoxicity

Cardiac toxic effects were first documented during early clinical evaluations of DOX, and in the late 1970s, first retrospective clinical studies showed convincingly that the observed cardiac disturbances could be directly attributable to repeated DOX administration. At the same time, these studies established the cumulative DOX dose received as the main risk factor of cardiotoxicity (Green et al., 2001; Lefrak et al., 1973; Von Hoff et al., 1979). Considering that more than 50% of long term-survivors of childhood cancer alone were treated with DOX or another anthracycline, anthracycline-induced cardiotoxicity is a widely prevalent problem that cannot be ignored. Namely, long-term survivors of childhood cancer are 8 times more likely than the general population to die from cardiovascular-related disease and, compared to sibling controls, they are 15 times as likely to suffer from heart failure (HF) (Lipshultz & Adams, 2010; Lipshultz et al., 1991). Despite very large numbers of new anthracycline compounds synthesized and tested, only few ones have been approved for clinical use, and none of them has fulfilled expectations of substantially improved cardiac safety. Therefore, a growing need to develop effective and cardioprotective strategies remains, in order to find a balance between the risks of cardiotoxicity and the benefits of oncologic therapy with DOX.

2.1 Clinical presentation and risk factors of doxorubicin-induced cardiotoxicity

DOX-induced cardiotoxicity is most often divided into 3 categories: acute changes, early-onset chronic progressive cardiotoxicity and late-onset chronic progressive cardiotoxicity (Aronson et al., 2006; Schimmel et al., 2004; Wouters et al., 2005). Acute DOX-induced cardiotoxicity occurs during DOX administration or immediately afterwards. It typically involves transient electrocardiographic abnormalities such as non-specific ST-T changes and QT prolongation, vasodilatation and hypotension. Pericarditis-myocarditis syndrome and ventricular dysfunction which manifest 1 to 3 days after the DOX treatment are extremely rare, and were more frequently seen in early trials using very high doses of DOX. Most often, all of the disturbances attenuate after discontinuation of the therapy. Early-onset chronic cardiotoxicity, such as cardiomyopathy which progresses to congestive heart failure (CHF) usually occurs within 1 year after discontinuance of DOX therapy. CHF may occur as total cumulative dosage of DOX approaches or exceeds 550 mg/m². Chronic cardiotoxicity reflects a progressive injury and loss of cardiac myocytes, with increasing cumulative DOX dose resulting in thinning of ventricular wall and decreased systolic performance. It is characterised by dilated (less often restrictive, mostly in children) cardiomyopathy, with subsequent development of left ventricular contractile dysfunction and congestive heart failure. Histopathological changes are quite unique and consist of distension of the sarcoplasmic reticulum of myocytes, cytoplasmic vacuolization, swelling of mitochondria and myofibrillar disarray and loss (Ferrans et al., 1997). Symptoms of the CHF include tachycardia, tachypnea, dilatation of the heart, exercise intolerance, pulmonary oedema, peripheral oedema and needs for treatment with diuretics and cardiac glycosides. These manifestations may respond to cardiac supportive therapy and may be self limiting, or may be irreversible and unresponsive to therapy and fatal (Aronson et al., 2006; Singal & Iliskovic, 1998; Sweetman, 2011; Wouters et al., 2005). Finally, late-onset chronic progressive DOX-induced cardiotoxicity occurs at least 1 year after the completion of therapy, sometimes even after decades of a prolonged asymptomatic interval. It has been suggested

that myocyte damage and ventricular dysfunction progress after the initial myocardial insult and may lead to late-onset cardiac decompensation. Although the DOX dose of 550 mg/m² was suggested to be relatively safe, CHF was found to begin at a lower cumulative dose in the presence of other risk factors, such as age above 70 years, combination therapy (cyclophosphamide, dacarbazine etc.), previous or concomitant mediastinal radiotherapy, history of pre-existing cardiac disease, or liver disease (Ludke et al., 2009; Singal & Iliskovic, 1998; Sweetman, 2011). Although sensitivity to DOX-induced cardiotoxicity exhibit large interindividual variation, the risk of developing impairment in myocardial function increases with increasing cumulative dose, occurring in 3-5, 5-8, and 6-20% in those receiving cumulative doses of 400, 450, or 500 mg/m², respectively, in schedules of rapid IV doses given every 3 weeks. The strong association between cumulative DOX dose and cardiotoxicity obviously is more important with increasing time after treatment (Lipshultz & Adams, 2010). Some clinicians suggest that late-onset cardiotoxicity can be clinically manifest in response to stressful situations (e.g. surgery, pregnancy), exercise and viral infection. Late-onset doxorubicin toxicity can be expected to be observed more frequently with the growing number of long-time survivors (survivors of childhood cancers) who have received DOX. Long term follow up shows that overt cardiac failure occurs from 0 to 16% of patients receiving anthracycline therapy. It depends on the cumulative dose, well recognized risk factor for late cardiotoxicity, radiation therapy involving the heart, age at diagnosis, greater length of follow up, higher dose rate and previous exposure to anthracycline (Kremer et al., 2002; McEvoy et al., 2010; Wouters et al., 2005). Therefore, recommendations for screening and management of late effects of therapeutic exposure used during treatment for pediatric malignancies are very important in every day practice.

2.2 Monitoring and markers of doxorubicin-induced cardiotoxicity

Clinical manifestations found on physical examination, and/or changes found on electrocardiographic (ECG) monitoring are not specific enough to diagnose DOX-induced cardiotoxicity. However, a persistent reduction in the voltage of the QRS wave is generally indicative of the need to perform further tests. Non-invasive cardiac monitoring, by means of serial echocardiography studies or radionuclide angiography, is useful in predicting the development of cardiomyopathy. However, since it may give normal results until damage is quite advanced, sensitivity may be improved with exercise stress tests (Singal & Iliskovic, 1998; Steinherz et al., 1992). Endomyocardial biopsy is the most sensitive indicator of cardiomyopathy, but it is limited by its invasiveness, need for histologic expertise, and costs. Therefore, there is need for simple methods, like serum and plasma markers to identify patients at risk. There is some preliminary evidence to suggest that concentrations of cardiac troponins and natriuretic peptides could be used as predictive markers of myocardial damage (Ludke et al., 2009; Schimmel et al., 2004). Anyway, according to the Childrens' Cancer Study Group (Steinherz et al., 1992), standard echocardiogram should be performed before the beginning of the treatment and at the third, sixth and 12th month after its completion. Subsequent exam should be repeated every 2 years for the rest of the life.

2.3 Prognosis and treatment

Historically, DOX-induced cardiomyopathies have a poor prognosis and mortality rate as high as 61% (Allen, 1992; Von Hoff et al., 1979; Wouters et al., 2005). It is refractory to

conventional therapy, therefore cardiac transplantation is the only solution associated with improved survival (Aronson et al., 2006; Sweetman, 2011). Just recent study reports that the response of DOX-induced cardiomyopathy to the current standard medical therapy is better, especially in patients treated with β -blockers. Nowadays, in the treatment of CHF, including DOX-induced cardiomyopathy, β -blockers and angiotensin-converting enzyme (ACE) inhibitors, are proven Class I medications according to the American College of Cardiology/American Heart Association and Canadian Cardiovascular Society guidelines (Hunt, 2005; Malcom et al., 2008).

2.4 Mechanisms of doxorubicin induced cardiac injury

Two important characteristics of doxorubicin induced cardiomyopathy are that is dose dependent and it is refractory to the commonly used therapeutic procedures (Lefrak et al., 1973; Singal & Iliskovic, 1998). Pathogenesis of cardiac injury caused by doxorubicin is multifactorial and it is in direct relationship with metabolism and antitumor activity of the drug (Figure. 1). Free radical stress, calcium overloading and mitochondrial dysfunction are main triggers in doxorubicin-induced cardiotoxicity. Subsequent gene expression changes and activation of the ubiquitin-proteasome system, cell death, as well as innate immunity activation all contribute to the toxicity (Shi et al., 2011). The level of the doxorubicin-induced oxidative stress is up to 10 times greater in the heart than in the other tissues (liver, kidney, spleen) (Davies & Doroshow, 1986; Doroshow & Davies, 1986; Gaetani et al., 1989; Lenzhofer et al., 1983; Milei et al., 1986; Mukherjee et al., 2003; Siveski-Iliskovic et al., 1995), and therefore oxidative injury of the heart is widely accepted theory presumed as a primary mechanism of the DOX-induced cardiotoxicity.

2.4.1 Role of iron and free radicals

Although doxorubicin-induced cardiotoxicity is multifactorial, formation of reactive oxygen species (ROS) has a leading role in promoting the oxidative myocardial damage. The high level of oxidative stress generated by anthracyclines is accounted to the molecular characteristics which allows drug to easily undergo redox reaction and subsequently free radical cascade.

Doxorubicin is belonging to the group of anthracycline antibiotics consist of naphthacenequinone nucleus linked through a glycosidic bond to an amino sugar, daunosamine (Figure. 2a). One-electron addition to the quinone moiety in ring C of DOX result in formation of a semiquinone that quickly regenerates its parent quinone by reducing oxygen to reactive oxygen species, like superoxide anion radical ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) (Figure. 2b).

This futile cycle is supported by a number of NAD(P)H-oxidoreductases [cytochrome P450 or -b5 reductases, mitochondrial NADH dehydrogenase, xanthine dehydrogenase, endothelial nitric oxide synthase (reductase domain) (Halliwell & Gutteridge, 2007; Minotti et al., 1999; Vasquez-Vivar et al., 1997). The dismutation of the $O_2^{\bullet-}$ is catalyzed by superoxide dismutase enzyme or it may occur spontaneously in the acidic environment - ($pH < 7$). Hydrogen peroxide is low toxic molecule, which is eliminated from the body by enzymatic antioxidative defence system (catalase -CAT and glutathione peroxidase - GPx). However, in the presence of the transition metals, especially iron, H_2O_2 and $O_2^{\bullet-}$ can

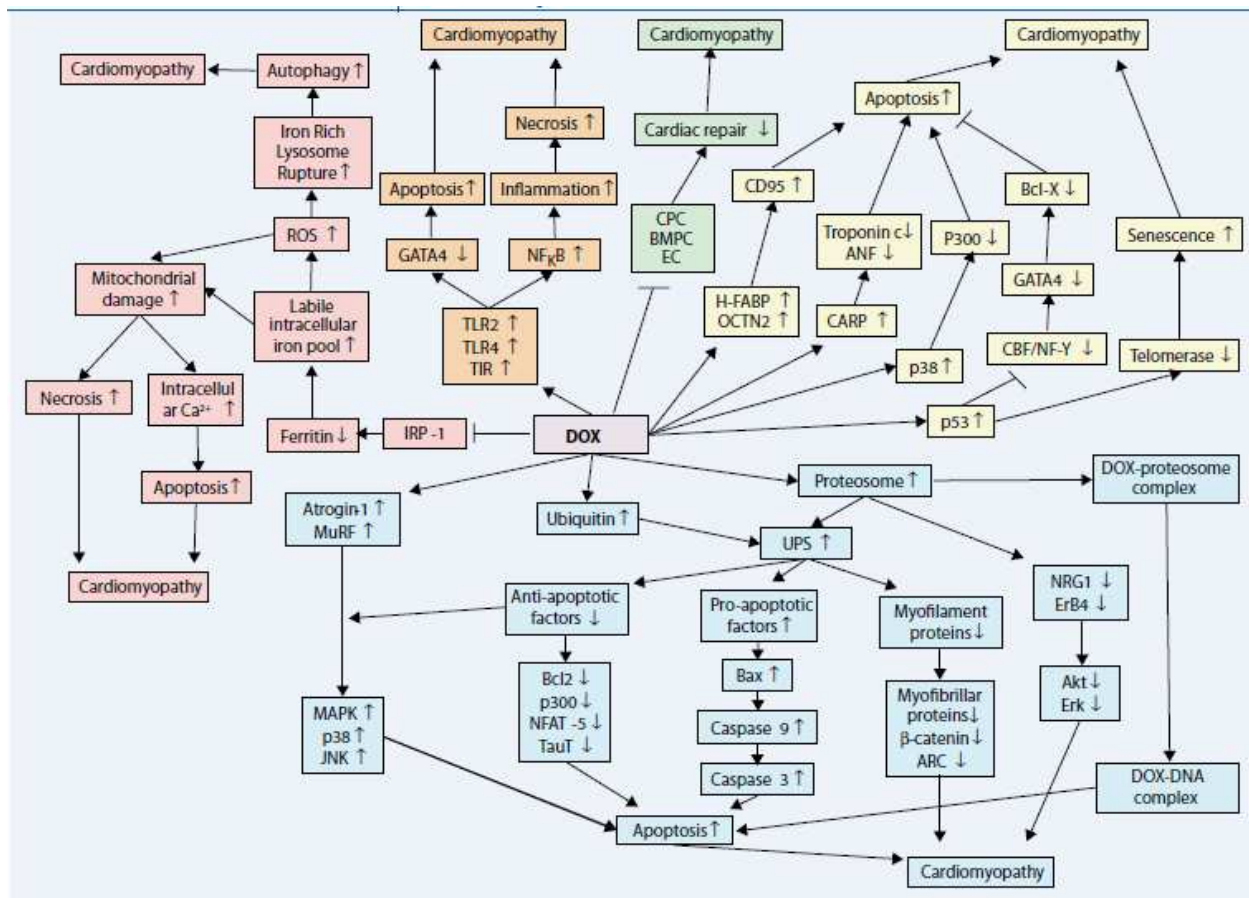


Fig. 1. A summary of potential mechanisms involved in doxorubicin (Dox)-induced cardiomyopathy as described in the text. The major mechanisms involve ROS and iron (red). Meanwhile, Dox-induced cell death (necrosis, apoptosis, and autophagy) and activation of innate immunity (brown), gene changes that reduce cardiac-specific gene expression or trigger apoptosis, induction of cardiac premature senescence in cardiomyocytes (yellow), activation of the ubiquitin-proteasome system (UPS) causing the balance of the protein system to shift toward pro-apoptosis, downregulation of prosurvival gene (NRG1 and ErB4) expression (blue), and impaired cardiac repair by inhibiting bone marrow, cardiac progenitor cell, and/or endothelial cell function (green) all emerge as potential mechanisms contributing to Dox cardiotoxicity. ROS reactive oxygen species, IRP iron regulatory protein, TLR toll-like receptor, TIR toll-interleukin-1 receptor, CARP cardiac Adriamycin-responsive protein, H-FABP heart fatty acid binding protein, OCTN organic cation/carnitine transporter, ARC apoptosis repressor with caspase recruitment domain, NFAT nuclear factors of activated T-cell, TauT taurine transporter, NRG neuregulin, ErbB epidermal growth factor receptor B, BMPC bone marrow cardiac progenitor cell, CPC cardiac progenitor cell, EC endothelial cell, arrows pointing up or down (inside text boxes) indicate increases or decreases in function or expression, horizontal arrows indicate positive regulatory sequence, blocks represent inhibition (Shi et al., 2011).

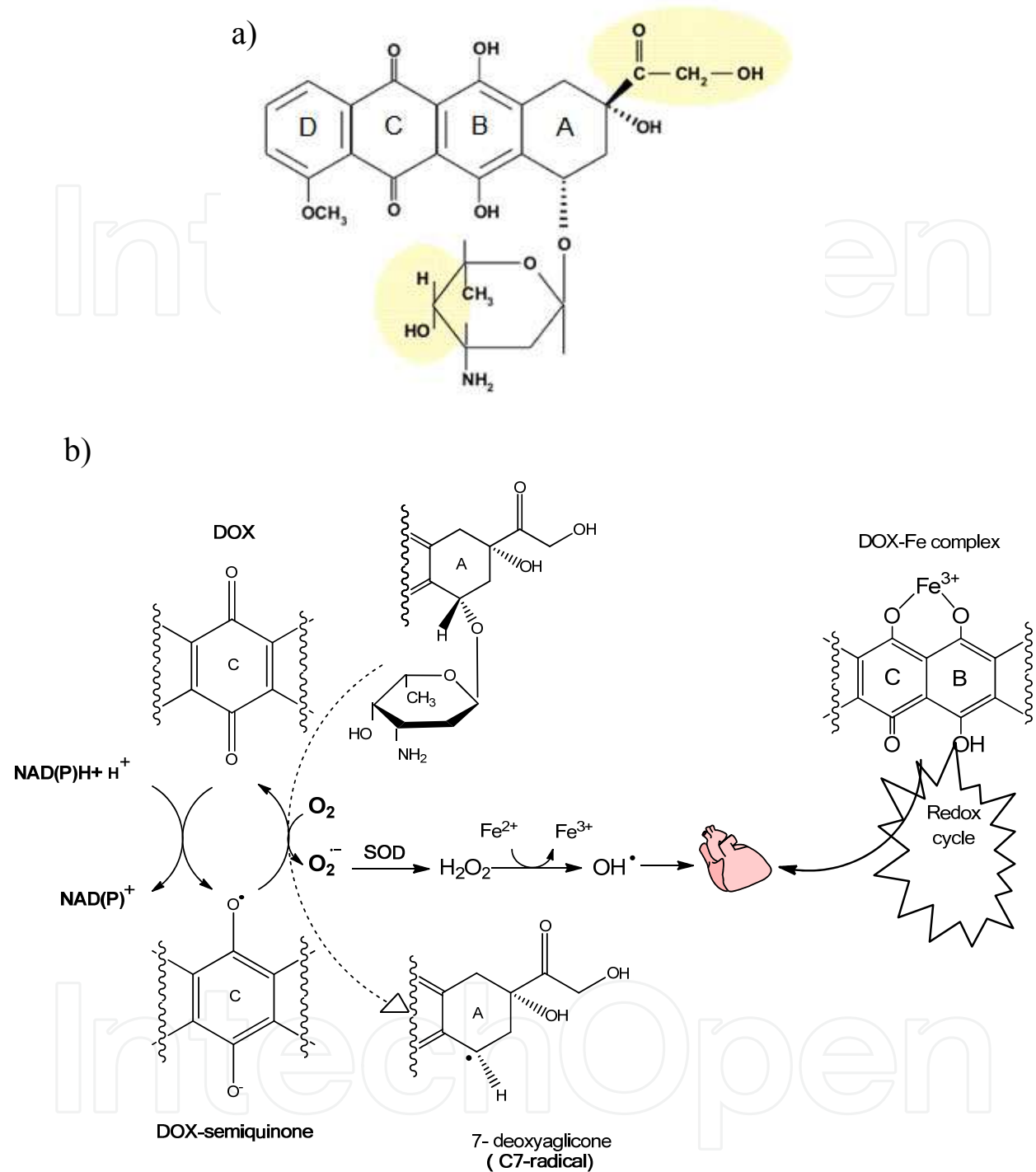
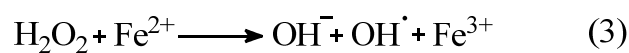
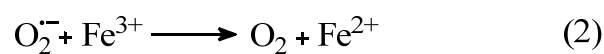
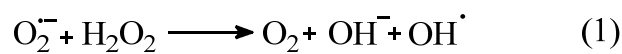


Fig. 2. Doxorubicin mediated redox cycling and free radical production



generate highly toxic hydroxyl radical (OH^\bullet). This process is occurring during the Haber-Weiss reaction (1) which is actually quite slow. Presence of iron strongly catalyzes formation of OH^\bullet , in two step process. In the first step ferric ion (Fe^{3+}) is reduced in to ferrous ion (Fe^{2+}) by $\text{O}_2^{\bullet-}$ (2). Ferrous ion than reacts with hydrogen peroxide (generated during DOX reduction) resulting with formation of hydroxyl radicals (Fenton reaction) (3).

Unlike hydrogen peroxide and superoxide anion radical, hydroxyl radical is extremely reactive and cannot be neutralized by antioxidative enzymes. Instead, OH^\bullet reacts with polyunsaturated fatty acids forming lipid peroxides, conjugated dienes and malonyl dialdehyde. As a consequence, the structure of lipid bilayer is modified resulting in cell membrane damage followed by cell dysfunction. In addition ROS can seriously affected proteins and nucleic acids, particularly ion channels and ion transporters (Halliwell & Gutteridge, 2007). Zhu et al. proved involvement of ROS in apoptosis in cardiac myocytes through p38 MAP kinase which is inhibited by CAT and SOD scavengers (Zhu et al., 1999). Cells have very little or no free iron available for catalyzing free radical reactions. However, studies have shown that redox cycling of doxorubicin directly increases the intracellular iron pool (Minotti et al., 1999; Xu et al., 2005; Xu et al., 2008). Formation of DOX semiquinone followed by $\text{O}_2^{\bullet-}$ generation is accompanied by iron releasing from ferritine. Ferritines are sphere shaped complex of 24 protein subunits with central cavity which can store up to 4500 atoms of iron. Eight channels through the sphere are lined by hydrophilic amino acid residues and six more are lined by hydrophobic residues. These transprotein channels provide the route for iron entry and exit from ferritin shell (Harrison et al., 1986; Theil, 1987). Anthracycline semiquinone is too large for entering the ferritin. However, superoxide anion is small enough to penetrate the shell and has reduction potential lower than that of polynuclear ferric oxohydroxide stored in the ferritin core. Combination of steric and thermodynamic factors thus enables $\text{O}_2^{\bullet-}$ to reach and reduce the iron pool of ferritin promoting the release of iron in its Fe(II) form through transprotein channels (Minotti, 1993; Minotti et al., 1999).

Furthermore, intracellular iron enrichment is mediated by doxorubicin and its metabolites. Cellular iron homeostasis is regulated by transferrin receptor (TfR) and ferritin. Both TfR and ferritin are mainly regulated at a post-transcriptional level involving interactions of iron regulatory protein (IRP-1) and iron responsive elements (IRE) in target genes (Xu et al., 2005; Xu et al., 2008). Namely, DOX can disrupt the Fe-S cluster of cytoplasmatic aconitase and inhibit IRP-1 whose role is to adapt levels of cellular iron according to metabolic needs.

The second mechanism by which Fe promotes oxidative injury involves generation of doxorubicin-iron complexes (DOX-Fe). Anthracyclines chelate Fe(III) forming the 1:1, 2:1 or 3:1 iron complex with association constant of 10^{33} (Figure. 2b) (Beraldo et al., 1985; May et al., 1980). In the presence of reducing agents (as NADPH cytochrome P450 reductase, glutathione or cysteine) DOX-Fe (III) complex are converting into DOX-Fe (II). This reaction come along with the formation of $\text{O}_2^{\bullet-}$ and conversion of quinone form of DOX to semiquinone free radical. Products of this reaction are further involving in the iron catalyzed Haber-Weiss reaction resulting in production of highly aggressive hydroxyl radicals (eq. 1-3). The semiquinone radical can be transform into C7 deoxydaglicone which is the potent alkylating agent.

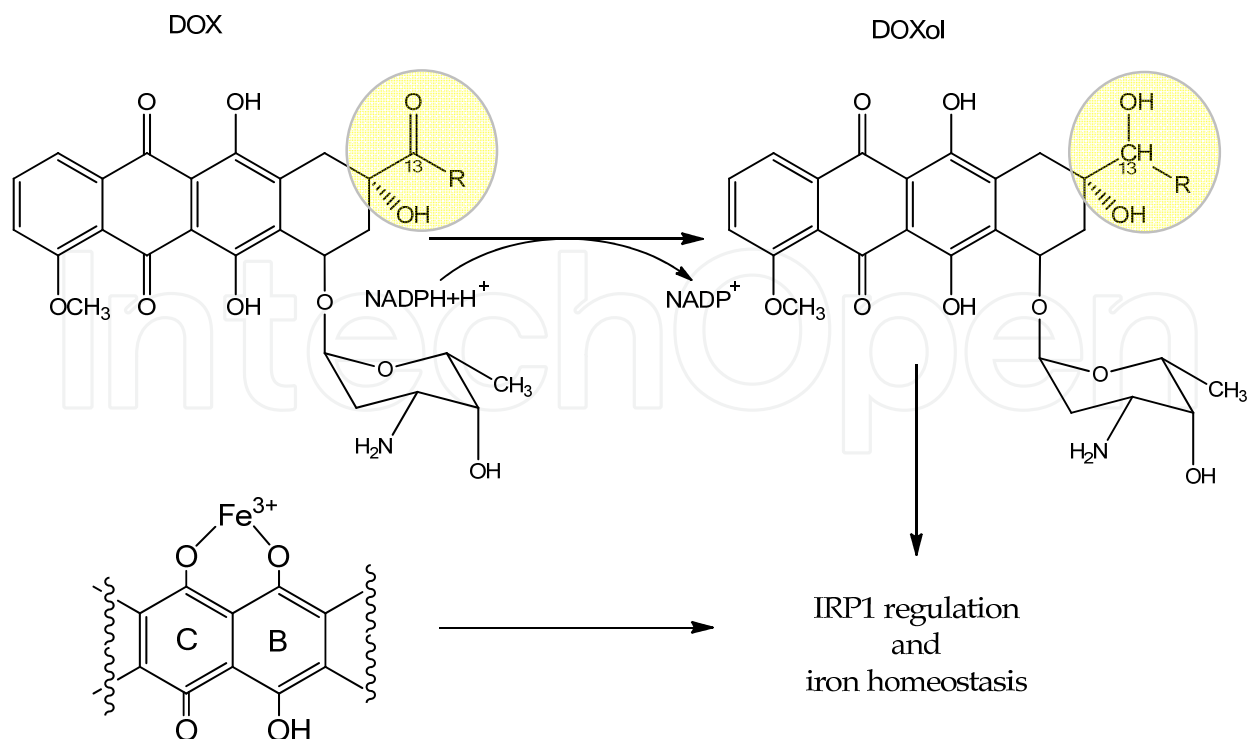


Fig. 3. Hypothesized mechanism of chronic cardiotoxicity mediated by DOXol and/or DOX-Fe complex

Different pathway of doxorubicin toxicity, other than oxidative injury, is involved in cardiac toxicity of the drug. Doxorubicinol (DOXol) is hydroxy metabolite of DOX formed upon two electron reduction on the C-13 carbonyl group of doxorubicin (Figure 3). This reaction is catalyzed by NADPH dependent carbonyl reductases (Forrest et al., 1991). DOXol is redox inactive and therefore not involved in free radical production. Notwithstanding, DOXol is involved in iron homeostasis (Licata et al., 2000; Minotti et al., 2001), myocardial energy metabolism, ionic gradients and calcium movements, impairing cardiac contraction and relaxation (Boucek et al., 1987; Olson, R. D. et al., 1988). Moreover, conversion of doxorubicin into DOXol significantly increases polarity of the parent drug molecule, thus favouring its retention within the cell. Considering the all aforesaid mechanisms of DOXol acting, it is logical conclusion that alcohol metabolite of doxorubicin is most likely responsible for the DOX induced chronic cardiomyopathy (Figure 3), while iron mediated free radical injury promote acute cardiotoxicity. This hypothesis is supported by findings of Miranda and co-workers which found that patients harbouring gene mutations in hereditary hemochromatosis show increased susceptibility to doxorubicin cardiotoxicity and exacerbated iron metabolism (Miranda et al., 2003). Numerous studies on transgenic mice with overexpressed antioxidants like catalase (Kang et al., 1996), superoxide dismutase (Abou El Hassan et al., 2003; Loch et al., 2009; Yen et al., 1996) and metallothioneine (Kang, 1999; Kang et al., 1997; Merten et al., 2005; Sun & Kang, 2002; Sun et al., 2001) have shown beneficial against DOX induced cardiotoxicity, confirming strong involvement of free radicals in oxidative injury of the heart.

The high oxidative metabolism and the poor antioxidative defence, compared with other organs, most likely explains why the doxorubicin is selectively toxic to the heart. Compared

to the liver, the cardiac antioxidant defense system is moderate. There is 150 times less catalase and four times less superoxide dismutase in the heart (Doroshov & Davies, 1986; Doroshov et al., 1980). In addition, the unique structure of cardiomyocytes, in which 50% of the cell organelles are mitochondria, serving both as a source and target for ROS, may explain why anthracycline antibiotics are selectively toxic to the heart (Berthiaume & Wallace, 2007). Cardiac mitochondria possess a NADH dehydrogenase on the outer surface of the inner mitochondrial membrane. Reduction of an anthracycline to the corresponding semiquinone by this enzyme produces an extremely high level of oxidative stress because the anthracycline transfers an electron to molecular oxygen and forms superoxide radicals. In relation to oxidative reactions, it must be emphasized that doxorubicin possess high affinity for cardiolipin, cardiospecific phospholipid that is rich in polyunsaturated fatty acids also placed in inner mitochondrial membrane. This high affinity, besides accumulation of DOX in interior of cardiomyocytes, also enables formation of DOX-cardiolipin complexes which can act as the substrate for the initiation of lipid peroxidation (Goormaghtigh & Ruyschaert, 1984). Once initiated, peroxidation continues autocatalytically, and has a progressive course that results in structural and functional changes in the heart tissue. The ultimate damage to mitochondria is oxidative damage of the mitochondrial DNA, which interferes with the regenerative capacity of the organelle. Once this irreversible damage occurs, the cardiomyocytes are destined to undergo apoptosis or necrosis, an event that may not occur until months or years after chemotherapy is completed (Davies & Doroshov, 1986; Doroshov et al., 1980). Therefore, the most significant pathological changes appear in the heart.

It is evident that there are still unknowns about exact mechanisms of the toxic effects of doxorubicin and its metabolites. In spite numerous investigations at the molecular level, iron mediated free radical injury and alteration in intracellular iron homeostasis have a key role in cardiotoxicity of anthracycline antibiotics and therefore considered classical mechanism underlying cardiac impairment induced by DOX.

2.5 Strategies for cardioprotection

Today's oncologists must be fully aware of cardiovascular risks to avoid or prevent adverse cardiovascular effects. The primary goal in the prevention is to minimise cardiac toxicities and to maximise oncological efficacy (Dorr, 1991; Schimmel et al., 2004). Currently, several preventive measures, such as: limiting cumulative dose of DOX, altering its administration, using new anthracycline analogues; combination with protective drugs and nutritional supplements are used.

2.5.1 Limiting cumulative dose of doxorubicin

Before the association between higher cumulative dose of DOX and the greater risk of cardiotoxicity was established, cumulative DOX doses greater than 400 mg/m² were administered to children with acute lymphoblastic leukaemia (ALL). Unfortunately, progressive cardiotoxic effects, clinically important, continued years after the DOX treatment completion (Lipshultz et al., 1991; Lipshultz et al., 2005). As a result, cumulative doses of DOX given to high-risk children and adolescent with ALL were reduced to a cumulative dose no more than 360 mg/m². Later on, after median follow up of 8.1 year, on the basis of obtained results, the cumulative DOX dose for high-risk ALL patients were

further reduced to 300 mg/m² (Nysom et al., 1998). However, patients vary widely in sensitivity and these values represent relatively arbitrary choices on a continuum of risk: there is no single safe dose (Shan et al., 1996; Von Hoff et al., 1979). Studies of late-onset anthracycline cardiotoxicity in childhood cancer survivors indicate that doses of DOX as low as 100 mg/m² increase the risk of reduced fractional shortening and higher afterload, whereas a cumulative dose of 270 mg/m² increases the risk of such abnormalities 4.5-fold. Even at doses that produce no symptoms, subclinical myocardial damage may occur, and in children this may result in diminished cardiac reserve and heart disease in later life (Hale & Lewis, 1994; Lipshultz et al., 1991).

2.5.2 Altering doxorubicin administration

Alteration of the dosage schedule to weekly rather than three-weekly dosage, or the use of continuous infusion, has also been advocated as a way of reducing doxorubicin cardiotoxicity (Legha et al., 1982a; Lum et al., 1985; Torti et al., 1983). At the same time, it was believed that continuous infusion might provide some cardioprotective benefits, because long-term exposures to drugs at modest concentrations would be safer than a pulsed supply of the drug at higher concentrations. Therefore, it was incorporated in many pediatric protocols although the comparative efficacy of these dosage schedules in various cancers and long-term effects on the development of DOX-induced cardiotoxicity have not been established (Lipshultz et al., 2005; Lipshultz et al., 2010). However, recently published meta-analysis performed to clarify the risk of early and late cardiotoxicity of anthracycline agents in patients treated for breast or ovarian cancer, lymphoma, myeloma or sarcoma showed that DOX given as bolus significantly increased the risk of clinical and subclinical cardiotoxicity compared with continuous infusion.

2.5.3 Use of doxorubicin-analogues

Several anthracycline derivatives have been developed with the aim of reducing the inherent cardiotoxicity of this class of compounds, including esorubicin, quelamycin, rodorubicin, and detorubicin. However, although this strategy has had some success, it turned out that almost all these compounds exhibit some degree of cardiotoxicity (Aronson et al., 2006; Sweetman, 2011; Weiss, 1992; Wouters et al., 2005). Epirubicin, idarubicin and mitoxantrone are the most promising ones. In the recently performed meta-analysis epirubicin significantly decreased the risk of clinical cardiotoxicity OR 0.39 (0.16, 0.57; $p < 0.008$; $I^2 = 0.5\%$), as well as any cardiotoxic event compared with DOX (Smith et al., 2010).

2.5.4 New drug-delivery systems and doxorubicin application

A promising approach to increase the efficacy and decrease the side-effects of DOX is its binding to drug delivery system, such as liposomes, nanoparticles and different cancer-targeting systems. Among the structural modifications of DOX, the liposome-encapsulated, is the one of the most promising cardioprotectants (Ludke et al., 2009). Liposomes are preferentially taken up by tissues enriched in phagocytic reticuloendothelial cells and with a sinusoidal capillary system like the liver and spleen. The continuous capillaries containing tissues, like heart muscle hardly take up liposomes, but it is not the case with the leaky capillary system of tumor site. Thus, the changes in tissue distribution of liposomal DOX

lead to less drug exposure in sensitive organs. The ability to reduce the plasma levels of free DOX is probably the source of the reduced cardiotoxicity provided by its liposomal formulation (Fulbright et al., 2010). Also, the release of drug is slow, which may avoid high peak plasma concentrations. It exists both in pegylated liposomal and, at the moment, these two formulations are approved for clinical use by the US Food and Drug Administration (Ludke et al., 2009; Sweetman, 2011). Several randomized clinical trial in adults have examined the activity and cardiotoxicity of liposomal DOX. Most of them indicated that the activity of liposomal DOX is similar to that of conventional, whether given alone or in combination with other drugs. At the same time, the risk of cardiotoxicity is markedly lower with liposomal DOX (Gianni et al., 2008; McEvoy et al., 2010; Safra, 2003; Smith et al., 2010). However, although clinically approved, conventional DOX continues to be the major drug in antineoplastic combinations used in the management of most solid tumors. Nanoparticles made of biodegradable polymers are particles with sizes in range from 20 to 200 nm that can encapsulate hydrophilic or hydrophobic drugs. Drugs delivered by nanotechnology delivery systems have a prolonged time in the circulation (Ferrari, 2005). Although carbon nanotubes and gold nanoparticles have demonstrated potential as a new drug delivery system, clinical use has been restricted due to concerns regarding their biodegradability (Liu et al., 2008). These particles should link with tumor-targeting ligands, such as peptides and small molecules. At the moment, DOX nanoparticles shows great promise in improving the oral bioavailability and reducing cardiotoxicity, but so many issues wait to be resolved in the future (Kalaria et al., 2009).

2.5.5 Combination with protective drugs

Apart from cumulative dose limitations, several attempts have been made to develop chemoprotectants to prevent the cardiotoxicity of DOX, without attenuating its antitumor effect. Following the aforementioned free radical hypothesis, antioxidants used as free radical scavengers have been examined both in experiments, and in clinical trials. On the other hand, considering a very important role of iron and the DOX-iron complex, iron chelators have also been used for prevention of DOX-induced cardiotoxicity (Dorr, 1991; Links & Lewis, 1999; Simunek et al., 2009).

3. Dexrazoxane

One of the most examined protectors against DOX cardiotoxicity is dexrazoxane (DEX). Activity of DEX against DOX-induced cardiotoxicity may be attributable to the intracellular conversion of DEX to an open-ring derivative ADR-925 which chelates iron, since one means of generating oxygen-free radicals may involve intramolecular reduction of DOX-iron conjugate. The cardioprotective activity of DEX is thought to result primarily from chelation of free and/or DOX-bound ferric ions in the myocardium by ADR-925 and some other hydrolysis products of DEX (Dorr, 1996; Hasinoff, 1989). As a result, the pool of free iron in the myocardium is reduced and bound iron is displaced from its potentially damaging complexes with DOX, thus preventing the formation of superoxide radicals after redox recycling. DEX has been shown experimentally to reduce DOX-induced cardiotoxicity in mice (Alderton et al., 1990); in rats (Della Torre et al., 1999; Herman et al., 1994; Villani et al., 1990); in beagle dogs (Herman et al., 1988) and in miniature swine (Herman & Ferrans,

1983). Treatment with DEX not only significantly reduced the incidence of cardiac lesions, but also increased the survival rate in animals treated with anthracyclines compared with anthracycline-only treated controls. Actually, DEX allowed administration of significantly larger cumulative doses of DOX, that otherwise would have been lethal in these animal models (Wiseman & Spencer, 1998). Studies in rats and beagle dogs have shown that DEX is generally more effective when administered shortly before or simultaneously with, rather than after DOX. In randomized, controlled trials, DEX provided effective cardioprotection in both adults and children receiving DOX chemotherapy for various malignancies (Lipshultz et al., 2004; Lopez et al., 1998; Speyer et al., 1992; Swain et al., 1997; Venturini et al., 1996; Wexler et al., 1996). In recent meta-analysis, data on clinical heart failure from eight trials with a total of 1561 patients were collected (van Dalen et al., 2011a). There were 11 cases of clinical heart failure among 769 patients randomised to DEX and 69 among 792 randomised to the control group. The meta-analysis showed a benefit in favour of DEX use (RR 0.18, 95% CI 0.10 to 0.32, $P < 0.00001$). In another 6 randomized clinical trials which evaluated cardioprotective agent DEX, four on women with advanced breast cancer, one with young people with sarcoma and one with breast cancer or sarcoma DEX given with DOX significantly reduced the risk of clinical cardiotoxicity, subclinical cardiotoxicity and any cardiotoxic event compared with DOX with no cardioprotective agent (Smith et al., 2010). Data on response rate (defined as the number of patients in complete and partial remission) could be extracted from six trials with a total of 1021 patients. These trials used comparable criteria to assess tumour response. There were 223 complete and partial responses among 503 patients randomised to DEX and 260 among 518 randomised to the control group. Meta-analysis showed no significant difference between the treatment groups. However, an important question regarding any cardioprotective intervention during DOX therapy is whether the cardioprotective drug could decrease the heart damage by anthracyclines without reducing the anti-tumour efficacy and without negative effects on toxicities other than cardiac damage. At the moment, despite its clear cardioprotective effects, DEX is not routinely used in clinical practice. This might be explained by the suspicion of interference with anti-tumour efficacy (that is response rate and survival) and by the occurrence of secondary malignant disease. However, meta-analyses of DOX anti-tumour efficacy and the appearance of secondary malignant diseases showed no significant difference between patients who were treated with or without DEX (van Dalen et al., 2011a). This latter finding was also identified in a recent publication (van Dalen et al., 2011b). A meta-analysis including three of the four randomised trials available on secondary malignancies after DEX, did not show a significant difference in the occurrence of secondary malignancies between children treated with or without DEX (RR 1.16, 95% CI 0.06 to 22.17, $P = 0.92$). The protective effects of DEX against DOX-induced cardiotoxicity are further supported by studies in children with leukemias and lymphomas, as well as with other malignancies (Lipshultz et al., 2010; Testore et al., 2008). However, further research is needed to fully understand the subtle risks associated with the use of DEX, as well as which methods of DEX administration are most efficient, and which doses are necessary to achieve adequate protection in children. At the moment, DEX is the only cardioprotective agent with proven efficacy in cancer patients receiving DOX. DEX is approved in many countries, including the United States, Canada, and a number of European countries. Current guidelines support the use of DEX for patients with metastatic breast cancer who have received more than 300

mg/m² of DOX in metastatic setting and who may benefit from continued DOX-containing therapy; treatment of patients who received more than 300 mg/m² in the adjuvant setting should be individualized, with consideration given to the potential for DEX to decrease response rates as well as decreasing the risk of cardiac toxicity. It should not be used for patients with metastatic breast cancer receiving initial DOX-based chemotherapy (Hensley et al., 2009; McEvoy et al., 2010; Sweetman, 2011). There is insufficient evidence to make recommendation for the use of DEX in the treatment of pediatric malignancies. It can be considered in adult patients who have received more than 300 mg/m² of DOX-based therapy for patients with advanced or metastatic cancer who have previously received anthracyclines. However, since DEX may potentiate haematological toxicity induced by chemotherapy, or the results for these adverse effects are ambiguous, for each individual patient a physician should balance the cardioprotective effects of DEX against the possible risk of adverse side effects (Ludke et al., 2009; van Dalen et al., 2011a). In addition, DEX appears equally well tolerated when administered with DOX at either 10:1 or 20:1 dosage ratios (e.g. if 50 mg/m² of DOX is used, 500 mg/m² DEX should be given). A reconstituted DEX solution should be given intravenously by slow push or rapid-drip infusion from a bag, starting 15-30 minutes before DOX administration. The optimal treatment regimen and cost effectiveness of DEX, and its protective efficacy against late-onset cardiotoxicity in patients given DOX chemotherapy during childhood or adolescence, are yet to be determined (Cvetkovic & Scott, 2005). On September 6, 2007, the U.S. Food and Drug Administration approved Totect[®] 500 mg, (dexrazoxane hydrochloride for injection) for the treatment of extravasation resulting from i.v. anthracycline therapy, an uncommon but serious complication (Kane et al., 2008). From all these facts, it can be concluded that well-designed cardioprotective intervention, like usage of DEX, can be an effective option as a strategy for DOX-induced cardiotoxicity prevention. Moreover, our ability to develop drugs such as DEX depends on our understanding of the molecular mechanisms involved in both antineoplastic and cardiotoxic effects of DOX. As it has been already mentioned, the current prevailing hypothesis is that DEX exerts its cardioprotective effects by binding free Fe, loosely bound Fe and Fe complexed to DOX, thus enabling the prevention of site-specific oxygen radicals production that damages cellular components of the heart (Cvetkovic & Scott, 2005; Dorr, 1996; Hasinoff, 1989). However, none of the other Fe chelating agents examined (aroylhydrazone iron chelators, defepirone etc) has reached the high protective efficacy of DEX. On the other hand, ADR-925 has a lower affinity for Fe than other helators, so it can be concluded that the iron-chelating properties of a compound are not the main determinants of cardioprotective action (Kaiserova et al., 2007). In recent experimental study it was clearly shown that DEX exerted protective effect against chronic daunorubicin cardiotoxicity *in vivo* (rabbits). Its cardioprotectiveness was based on the rescue of cardiomyocytes not only from degenerative changes and non-programmed cell death, but also from programmed cell death (apoptosis), as well (Popelova et al., 2009). Actually, DEX was shown to block all the major apoptotic pathways, and, therefore, protection of cardiomyocytes did not seem to be primarily lipoperoxidation-dependent. Apart from all the aforementioned, it is already known that DEX binds directly to topoisomerase II and locks the enzyme in a stable and closed clamp conformation around DNA (Roca et al., 1994). Since, anthracyclines are, as already mentioned, topoisomerase II inhibitor, it has been proposed that DEX may have protective effects against such agents in this way (Lyu et al.,

2007). In addition, beta isoform of topoisomerase II is abundant in post-mitotic myocardium, including mitochondria (Wallace, 2007; Wang, 2002). It was also previously mentioned that DOX impairs calcium homeostasis in cardiomyocytes, not only affecting sarcoplasmic reticulum function, but also mitochondria (Minotti et al., 2004). The fact that anthracycline-induced calcium overload can be prevented with DEX, co-treatment gives one more explanation for its cardioprotective efficacy (Simunek et al., 2005). Namely, it was shown that systolic heart failure induced by chronic daunorubicin administration is primarily accompanied by persistent calcium overload of cardiac tissue and the protective action of DEX is associated with the restoration of normal myocardial Ca^{2+} content. Therefore, mechanisms other than the traditionally accepted "ROS and iron" hypothesis are involved in DOX-induced cardiotoxicity, and knowledge of them may be a better basis for designing approaches to achieve efficient and safe cardioprotection.

4. Amifostine

Amifostine is a simple aminothioliol compound that is a product of a developmental program initiated in 1959 by the United States Army, in studies conducted at the Walter Reed Institute of Research to identify and synthesize drugs capable of conferring protection to individuals working in radioactive environments (Dragojevic-Simic & Dobric, 1996; van der Vijgh & Peters, 1994). As a result of this program, over 4,000 compounds were synthesized and tested. Amifostine, code named WR-2721, emerged as the lead compound. The drug was modeled after experimental studies, which showed reduced bone marrow toxicity when the sulfur-containing amino acid cysteine was administered before total body irradiation. It was the first cytoprotective agent to be identified as being capable of differentially protecting nonmalignant (normal) versus malignant cells from the cytotoxic effects of ionizing radiation and some antineoplastic agents (Yuhas, 1979; Yuhas et al., 1980; Yuhas & Storer, 1969). Amifostine itself is an organic phosphorothioate that is inactive until it is dephosphorylated by alkaline phosphatase to yield the active free thiol (SH) form, code named WR-1065 (Yuhas, 1980). Selectivity, in terms of cytoprotection of normal tissue, preferentially, is believed to be related to differential distribution and absorption of the parent drug in better perfused normal tissues as well as greater alkaline phosphatase activity in normal tissues, related to the acidic pH values found in hypoxic tumor tissues (Calabro-Jones et al., 1988; Dragojevic-Simic & Dobric, 1996; Smoluk et al., 1988; Utley et al., 1984; Yuhas, 1980). Normal tissues, especially at the capillary level, have a higher specific activity of alkaline phosphatase which releases WR-1065 for rapid local uptake into normal tissues. In contrast, the activity of this enzyme in neoplastic capillaries is appreciably lower, a feature that contributes to this apparently selective uptake. It was also demonstrated that the rate constant for the uptake of WR-1065 across the cell membrane is accelerated with small differences in pH, favouring the pH of 7.4, which is found in normal tissues versus relative acidity in tumors (Calabro-Jones et al., 1988). Tissue distribution studies, performed in experimental animals, demonstrated the uptake of amifostine and its metabolite. Kidney, bladder, salivary gland and liver accumulate high level of drug; heart, small intestine and spleen accumulate it moderately, while the spinal cord, brain and tumours accumulate very little, if any (Utley et al., 1976; Washburn et al., 1976; Yuhas, 1980). The "ideal cytoprotector" should have: the ability to be administered before or concurrent with therapy, selectivity for

normal versus cancer cells, ability to prevent/reduce toxicities of chemotherapies, no adverse effects on therapeutic efficacy, effectiveness against a variety of therapy-associated toxicities, a tolerable safety profile and simple way of administration (Capizzi, 1999b; Mabro et al., 1999; Spencer & Goa, 1995). The aforementioned findings concerning amifostine prompted numerous preclinical and clinical studies and led to the ultimate marketing approval many years later (Hensley et al., 2009). The American Society of Clinical Oncology recommends amifostine for prevention of cisplatin-associated nephrotoxicity, reduction of grade 3 and 4 neutropenia (alternative strategies are reasonable), and to decrease acute and late xerostomia with fractionated radiation therapy alone for head and neck cancer. The current US Food and Drug Administration-approved dose of amifostine is 910 mg/m² intravenously over 15 minutes, 30 minutes before chemotherapy. Common toxicities include acute hypotension, nausea, and fatigue. When given with radiation therapy, the recommended amifostine dose is 200 mg/m²/day, as slow i.v. push over 3 minutes, 15 to 30 minutes before each fraction of radiation therapy. The hypotension associated with amifostine at this dose is less frequent, but still requires close monitoring (Hensley et al., 2009; McEvoy et al., 2010; Sweetman, 2011). As aforementioned, if administered before cytotoxic chemotherapy, amifostine provides cytoprotection of various normal tissues, with the exception of central nervous system, without attenuating its antitumor response (Capizzi, 1999a; Culy & Spencer, 2001; Dragojevic-Simic & Dobric, 1996; Kouvaris et al., 2007; Mabro et al., 1999; Spencer & Goa, 1995). It protects the bone marrow against both the harmful effects of ionizing radiation as well as cyclophosphamide, nitrogen mustard, melphalan, mitomicin C, carmustine, 5-fluorouracil, carboplatin and cisplatin. Protection from cisplatin nephrotoxicity and ototoxicity has been shown, as well as protection of peripheral neural tissue from cisplatin, paclitaxel, vincristine and vinblastine toxicity. However, data concerning amifostine efficacy in preventing the toxic effects of DOX are still insufficient (Bolaman et al., 2005; Dobric et al., 1998; Dragojevic-Simic et al., 2004; Nazeyrollas et al., 1999; Potemski et al., 2006). As previously mentioned, amifostine uptake has been documented to be relatively high in normal tissues compared with experimental tumors. Moreover, sixty minutes after amifostine injection, level in heart tissues was approximately sevenfold higher than in tumor and sixfold higher than in serum (Dorr, 1996; Yuhas, 1980). These tissue distribution studies suggest that apart from the bone marrow, salivary gland and kidneys, the heart might also benefit from cytoprotection provided by amifostine treatment prior to cardiotoxic radio- and chemotherapy. In our previous experiment in which general radioprotective efficacy of amifostine was examined in rats subjected to whole body irradiation (WBI, absolutely lethal dose of X-rays), it was shown that amifostine significantly protected rats and increased their survival comparing to the unprotected animals (Dobric et al., 2007; Trajkovic et al., 2007). Moreover, a mean cardiac damage (MCD) score, obtained by histopathological analysis, in amifostine-pretreated rats was significantly reduced compared with unprotected animals, on both days 7 and 28 after WBI. It has been supposed for a long time, according to data derived from the experiments with irradiated animals, that once inside the cell, protective effects of WR-1065 appear to be mediated by scavenging free radicals, hydrogen donation, induction of cellular hypoxia, the liberation of endogenous nonprotein sulfhydryls (mainly glutathione) from their bond with cell proteins, the formation of mixed disulphides to protect normal cells, etc (Brown, 1967; Grdina et al., 1995; Smoluk et al., 1988; Spencer & Goa, 1995). On the other hand, previous *in*

vitro studies demonstrated that amifostine, and especially WR-1065, was able to scavenge OH. and O₂⁻, including DOX-derived O₂⁻ generated by NADH respiration of heart mitochondria particles. Marzatico et al., (2000) showed that amifostine scavenging activity is exerted mainly against highly reactive OH. , the most dangerous reactive oxygen species from a biological point of view (Marzatico et al., 2000). It was also shown that both amifostine and WR-1065 protected cultured neonatal rat cardiac myocytes against DOX-induced loss of cell viability (Dorr et al., 1996). These results had clinical relevance since they showed that the exposure concentrations of amifostine and WR-1065 were limited to 2.0 µg/ml, which was 10% of the peak plasma level achieved in humans given intravenous amifostine 740 mg/m² (Shaw et al., 1988). Effects of amifostine on perfused isolated rat heart and on acute DOX-induced cardiotoxicity were also examined (Nazeyrollas et al., 1999). Amifostine induced coronary vazodilatation, and, when associated with cardiotoxic concentrations of DOX, displayed cardioprotective effects, but the mechanism of its action was not elucidated. Moreover, *in vivo* experiments demonstrated that selective cytoprotection against DOX-induced toxicity with amifostine and WR-1065 can be achieved without abrogating antitumor activity of DOX (Bhanumathi et al., 1994; Dorr, 1996; Spencer & Goa, 1995). In our own previous study (Dobric et al., 2003; Dragojevic-Simic et al., 2004) the efficacy of amifostine (75 mg/kg *ip*) in reducing the cardiotoxicity of DOX in Wistar rats given in a dose of 1.25 mg/kg *ip*, 4 times per week, for 4 weeks was examined. Amifostine was administered each time, 20 min. before DOX. Mortality, general condition and body weight of animals were observed, while evaluation of cardioprotective efficacy of amifostine was performed by analyzing the ECG parameters, response to pro-arrhythmic agent aconitine, as well as activity registration of the *in situ* rat heart preparations. The pretreatment with amifostine significantly reduced mortality of rats comparing with unprotected group and reversed the arrhythmogenic dose of aconitine to values not significantly different from the control ones. It also antagonized DOX depressive effects on heart contractility. Moreover, a MCD score, obtained by histopathological analysis, in amifostine-pretreated rats was significantly reduced compared with unprotected animals, 4 weeks after the last dose of treatment. Also, amifostine significantly decreased number of mast cells in the heart in DOX-treated rats. Although mast cells could also be seen everywhere in the myocardium, they were predominantly situated around the blood vessels, like in control. Some other investigations in which rats were subjected to relatively high, cumulative dose of DOX, also showed protective effects of amifostine (Herman et al., 2000; Nazeyrollas et al., 2003). Amifostin dose was in range from 7 to 200 mg/kg, depending on the experimental model employed. However, concerning the methodology used, it was not possible to give some solid evidence about the amifostine molecular mechanism of action. We supposed that amifostine protected cardiomyocytes plasmalemma owing to its scavenging activity, and influenced the duration of action potential, especially the recovery phase and Ca²⁺ movement across the cellular membrane (Dragojevic-Simic et al., 2004). This might have contributed to the reduction of subsequent signalling pathways caused by DOX, since its detrimental effect eventually led to myofibrillar degeneration. Although there are some other opinions, our own results, as well as results obtained by other authors, support the statement that acute and chronic toxicity of DOX share the same mechanisms and, accordingly, chronic toxicity arises from repeated episodes of acute exposure, inducing cumulative damage (Dragojevic-Simic et al., 2011a; Dragojevic-Simic et al., 2004; Jensen,

1986; Luo et al., 1997; Pelikan et al., 1986). Thus, not only formation of highly reactive oxygen species, especially OH \cdot , should be blamed for acute, as well as chronic DOX cardiotoxicity, but also their scavenging could be the main mechanisms of amifostine protection against both types of toxicities. According to Luo et al., (1997), reactive oxygen species, by inducing lipid peroxidation, after application of DOX produce cytotoxic aldehydes which result in inflammatory reactions (Luo et al., 1997). This eventually leads to increased synthesis of cytokines, infiltration of mononuclear cells and death of heart cells. In accordance with that, in our already described experiment (Dragojevic-Simic et al., 2004), the presence of mononuclear cells and fibroblasts was decreased in amifostine-protected rats compared with DOX-only treated group, and the necrotic cardiomyocytes were very rare. It has already been demonstrated that reactive oxygen species are increasingly produced by inflammatory cells in response to stimulation by cytokines such as TNF- α , IL-1, IL-6 and IL-17 and play an important role as messengers of the intracellular signaling pathways (Babbar & Casero, 2006; Jamaluddin et al., 2007). It was suggested that ROS, in turn, activate inflammatory cells that have part in the progression of inflammation. Therefore, targeting ROS may have a therapeutic value as a strategy to reduce the development of inflammation. It was already shown that amifostine, given in doses of 186 mg/kg *per os*, showed significant reduction of paw oedema in the carrageenan-induced paw inflammation in mice, comparable to that achieved by aspirin (Bhutia et al., 2010). Moreover, in our previous experiments amifostine reduced carrageenan-induced rat paw oedema achieving high degree of anti-inflammatory activity. Given in doses of 100, 200 and 300 mg/kg, *ip* 30 min before carrageenan challenge, it significantly reduced footpad swelling being the most effective in the highest dose tested (Dragojevic-Simic et al., 2011b). Therefore, anti-inflammatory effects of amifostine can contribute to its protective effects in DOX-induced cardiotoxicity. On the other hand, in our previous experiments amifostine provided significant protective effects against early toxic changes in rat heart induced by single high doses of DOX (6 and 10 mg/kg *iv*) (Dobric et al., 1998; Dragojevic-Simic et al., 2007; Dragojevic-Simic et al., 2011a). Amifostine (300 mg/kg *ip*, 30 min, before DOX) successfully prevented significantly increased activity of CK, AST, LDH and α -HBDH enzymes, as well as malondialdehyde (MDA) level in the serum of animals treated with DOX in a dose of 6mg/kg. In a similar experimental model Bolaman et al (2005) showed that MDA level was lower, and glutathione and catalase levels were higher in the hearts of amifostine-pretreated group of animals comparing to unprotected rats (Bolaman et al., 2005). In our experiment (Dragojevic-Simic et al., 2011a) the application of amifostine in rats treated with DOX dose of 10 mg/kg reduced MCD score to the value obtained in the group of rats treated with 6 mg/kg of DOX, only. The ultrastructural analysis (UA) showed that pretreatment with amifostine in rats which received 6mg/kg of DOX successfully protected sarcolemma of cardiomyocytes, while the mitochondria damage in the protected group was far less prominent. Capillaries were less morphologically changed and apoptosis of endothelial cells was extremely rare in comparison with non-treated animals. The UA of the rat heart 48 hours after administration of 6mg/kg of DOX in this experiment revealed cardiomyocyte alterations described as *oncosis*. This is in accordance with the results of other authors, who also showed that the earliest and most often changes in the rat heart after application of high doses of DOX were cellular oedema and swelling of the mitochondria in cardiomyocytes. On the other hand, low-dose DOX exposure induced apoptosis of these cells (L'Ecuyer et al., 2004; Olson, H. M. & Capen, 1977; Simunek et al., 2009; Zhang, Y. W. et al., 2009). Our findings concerning prominent protection of mitochondria with amifostine are in line with

widely accepted hypothesis that mitochondria are a primary target of DOX-induced oxidative stress (Berthiaume & Wallace, 2007; Grdina et al., 2002). It is also known that amifostine is negative charged thiol and this protector accumulates within the mitochondria and around DNA, explaining higher protective amifostine potential for them than neutral or positive charged thiols, especially since some studies using perfused rat hearts showed that DOX is localized primarily to the nucleus and mitochondria of the cell (Berthiaume & Wallace, 2007). It was also shown that both amifostine and WR-1065 significantly reduced DOX-induced heart cell toxicity, measured by ATP content normalised to total cellular protein (Marzatico et al., 2000). This finding can also be explained by effective protection of mitochondria, as in our study, since oxidative phosphorylation is one of the functions of this organelae which provides a substantial portion of the ATP needed to meet energy demands in the heart. On the other hand, as already mentioned, several lines of evidence suggest that amifostine is presumably modified into WR-1065 by membrane-bound alkaline phosphatase, highly expressed in the endothelium, It quickly penetrates into cells, where it acts as free-radicals scavengers and protects cells from oxidative damage (Calabro-Jones et al., 1988; Dragojevic-Simic & Dobric, 1996; Smoluk et al., 1988; Utley et al., 1984; Yuhas, 1980). Potent protective effects of amifostine pretreatment in the model of lipopolysaccharide (LPS) -induced lung injury *in vivo* and attenuation of pulmonary endothelial cell barrier dysfunction *in vitro via* attenuation of oxidative stress, inhibition of redox-sensitive MAP kinases, NF- κ B inflammatory cascade, as well as attenuation of LPS-induced cytoskeletal remodeling and disruption of endothelial cell adhesions leading to the preservation of endothelial cell monolayer integrity were shown (Fu et al., 2009). On the other hand, marked elevation of the expression of antioxidant gene manganese superoxide dismutase (MnSOD) in human microvascular endothelial cells following their exposure to a WR-1065 can result in elevated resistance to the cytotoxic effects of ionizing radiation. Namely, MnSOD is nuclear-encoded mitochondrial enzyme that scavenges $O_2\cdot^-$ in mitochondrial matrix, and has been shown to be highly protective against radiation-induced ROS (Murley et al., 2006). Based on the current data, we speculate that succesful amifostine protection of DOX-induced damage of heart capillaries, whose endothelium, as a rich source of oxidants, contributes a lot to the oxidant-rich environment at that locus in this model (Dobric et al., 1998; Dragojevic-Simic et al., 2007; Dragojevic-Simic et al., 2011a), may be mediated by its antioxidant properties resulting in downregulation of oxidative stress and redox-sensitive signalling cascades. It is obvious that mechanisms of amifostine protection against DOX-induced cardiotoxicity, different from traditionally emphasized "scavenging free radicals" are also involved in amifostine-induced cardioprotection. Therefore, not only further preclinical and clinical studies are needed in order to implement amifostine in everyday oncological practice more succesfully, but also they should enable further progress in elucidation of DOX cardiotoxicity mechanisms, which represent a perpetual enigma.

5. Fullerenol $C_{60}(OH)_{24}$

With discovery of fullerene, 25 years ago (Kroto et al., 1985), started new era in chemical sciences. Fullerene, the third carbon allotrope, is a classical engineered material with the potential application in biomedicine. Spherical fullerene C_{60} , known as buckyball, is the most representative member of the fullerene family. With the shape of an icosahedron, containing 12 pentagons and 20 hexagons (Hirsch & Brettreich, 2005; Kratschmer et al.,

1990), C_{60} become symbol of symmetry in chemistry and important member of nanomaterials family (Figure. 4). Unique physical and chemical properties, made backyball to quickly find its application in the material science, electronics and nanotechnology.

The biological activities of fullerenes are considerably influenced by their chemical modifications and light treatment. The most relevant feature of fullerene C_{60} is the ability to act as a free radical scavenger. Properties attributed to the delocalized π double bond system of fullerene cage allow C_{60} to quench various free radicals more efficiently than conventional antioxidants. The chemical modification of fullerenes by adding the OH groups to their carbon surface yields a variety of polyhydroxylated structures $C_{60}(OH)_x$ exhibiting different degrees of solubility and antioxidant activity in the aqueous environment (Xing et al., 2004; Zhang, Jian-Min et al., 2004). Polyhydroxylated derivate of fullerene - fulleranol ($C_{60}(OH)_{24}$) was synthesized in alkaline media by complete substitution of bromine atoms of $C_{60}Br_{24}$ with hydroxyl groups (Figure. 5). New polyhydroxylated derivate completely maintains symmetry of parent C_{60} molecule (Djordjevic et al., 2005). Combination of the moderate electron affinity of fulleranol and their allylic hydroxyl functional groups makes fulleranol a suitable candidate for water-soluble antioxidants in biological systems.

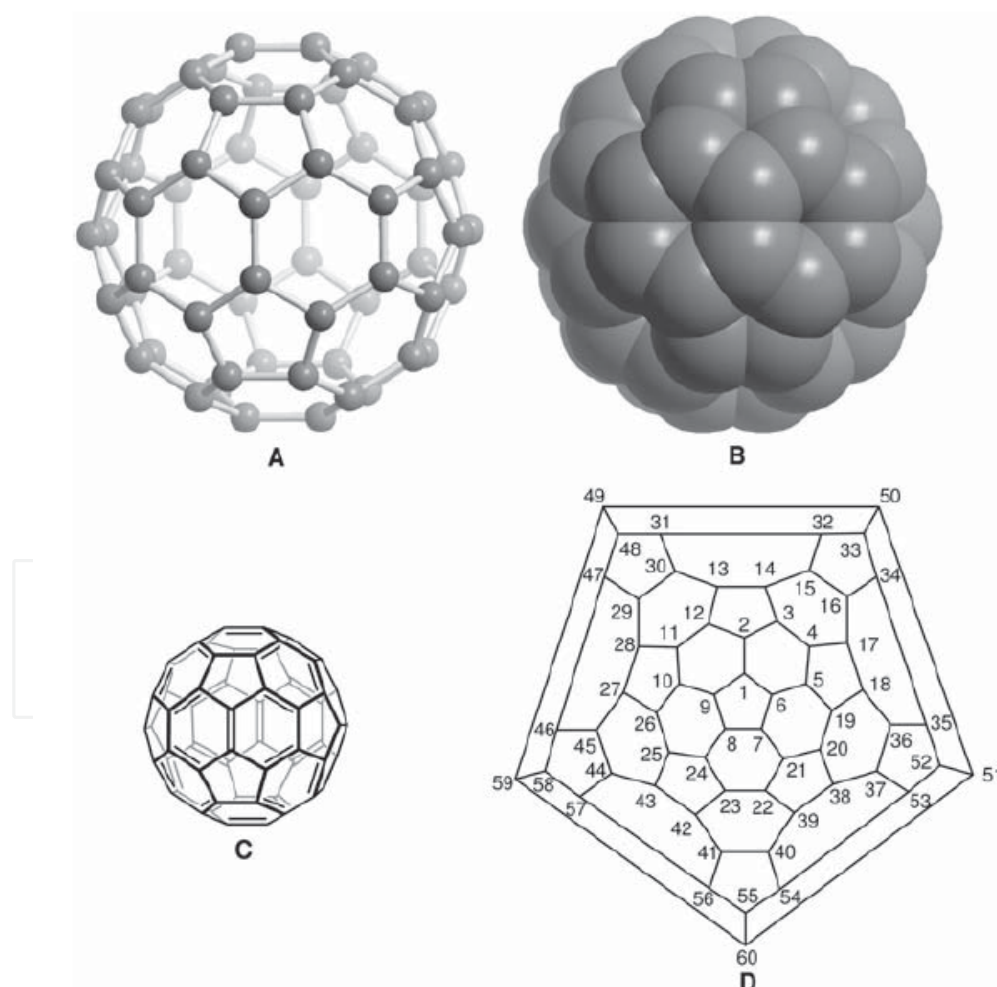


Fig. 4. Schematic representations of C_{60} . (A) ball and stick model, (B) space filling model, (C) VB formula, (D) Schlegel diagram with numbering of the C-atoms (Hirsch & Brettreich, 2005).

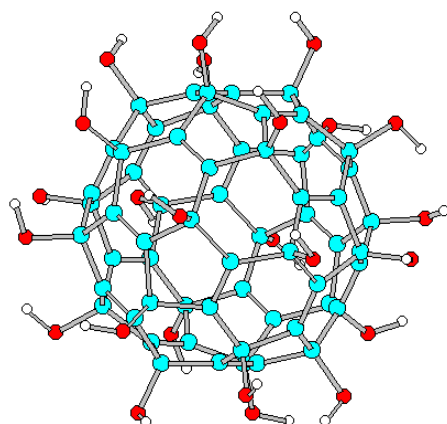


Fig. 5. Molecular structure of fulleranol

Fullerenol $C_{60}(OH)_{24}$ exhibited antioxidative activity in nanomolar concentrations in dose dependent manner against stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical and hydroxyl radical ($OH\cdot$) during the Fenton reaction using electron spin resonance (ESR) spectroscopy. Higher concentrations of fulleranol (0.71 - 0.88 mmol/L) stronger suppress production of hydroxyl radical than DPPH radical (Djordjevic et al., 2005). Authors suggested two possible mechanisms of antioxidative activity of fulleranol: (i) radical addition reaction of $2n$ OH radicals to remaining olefin double bonds of fulleranol core to yield $C_{60}(OH)_{24}+2n$ OH ($n=1-12$) and (ii) simultaneous hydrogen atom donation to DPPH and OH including the formation of relatively stable fulleranol radical $C_{60}(OH)_{23}O$ (Figure. 6.) (Djordjevic et al., 2005). These mechanisms are not mutually excluded. Studies of antioxidative activity of fulleranol, imply that fulleranol $C_{60}(OH)_{24}$ has the ability to act both as an iron chelator and as a direct free radical scavenger. Study with hydroxyfullerene and metal salts demonstrated that fullerenols react rapidly and irreversibly with variety of metal salts within the pH range of 3.0-8.5, forming insoluble metal-hydroxyfullerene cross-linked polymers (Anderson & Barron, 2005).

Extensive *in vivo* studies on antioxidative protection of fulleranol against DOX induced toxicity were done. It was found that pretreatment with 100 mg/kg of fulleranol, succeed to abolish acute toxic effects on the heart, caused by single dose application of doxorubicin (10 mg/kg i.p.) (Milic Torres et al., 2010). Previous studies have shown that application of doxorubicin causes damage of the heart and baroreceptors, which is indicated by reflex alterations (Rabelo et al., 2001). Results of this study showed that in DOX treated animals the reflexes were maintained, but their appearance was delayed, which implies the presence of some heart damage. Fulleranol in both applied doses (50 and 100 mg/kg i.p.) almost completely annulated this DOX induced disturbance and the time for appearance of adrenaline-induced reflex bradycardia in ECG record was in the level of control values. The most intensive cardiac histopathological changes, induced by DOX, were noticeable by light microscopy on the 14th day after the treatment. Fulleranol applied as a pretreatment, in dosage of 100 mg/kg, exerted protection and sustained structural integrity of the cardiac cells in DOX treated animals (Figure. 7.). Fulleranol, applied alone, in dosage of 100 mg/kg caused mild vascular changes, which are likely to be reversible. Both applied dosages (50 and 100 mg/kg) used as a pretreatment, maintained the majority of investigated parameters (biochemical and parameters of oxidative stress) in the level of control values.

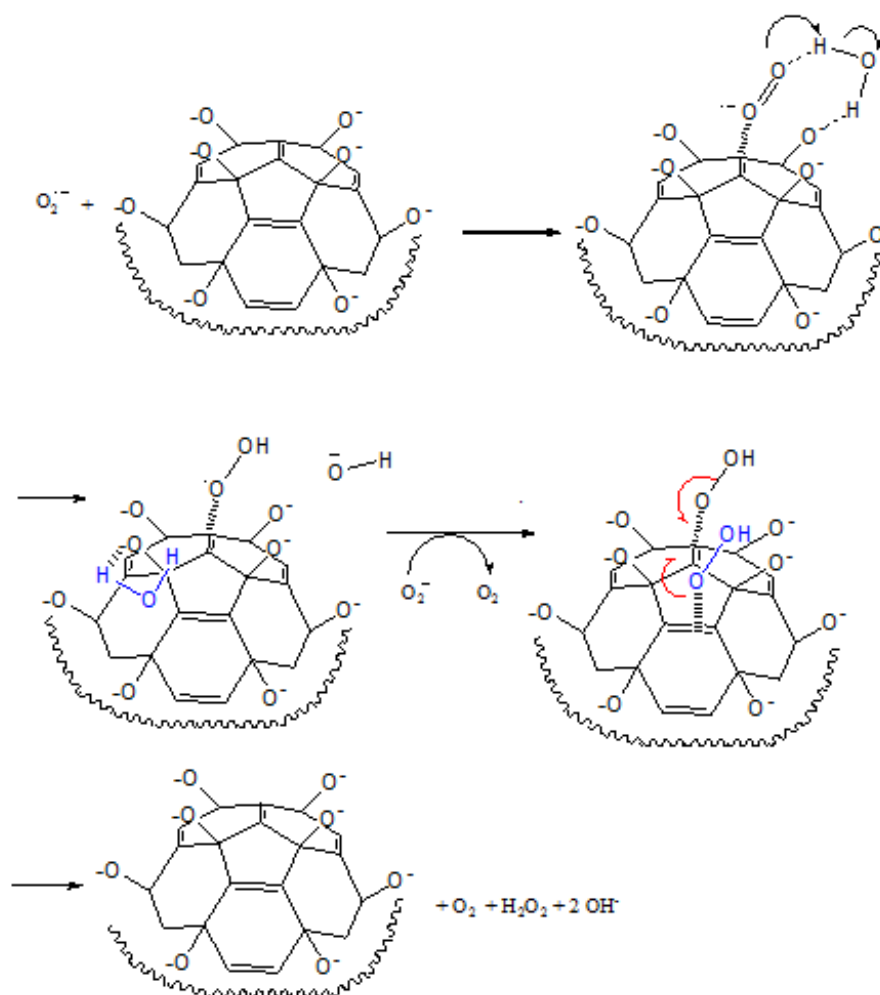


Fig. 6. Hypothesized mechanisms of fullereneol antioxidant activity

This indicates the protective effect of polyhydroxylated fullerene. Results of work Milic Torres et al., confirm the hypothesis and complete the previously conducted investigation that indicated that fullereneol possess high antioxidative and cytoprotective potential, without any recorded side effect. Considering mechanisms of DOX toxicity (predominantly based on free radical production), previous investigation and results obtained in our experiment, it can be concluded that protective role of fullereneol is established on its high antioxidative potential. Regarding to unique electrochemical features, fullereneol exerts its antioxidant effect acting as a free radical sponge and/or removing of free iron through formation of fullereneol-iron complex and therefore enable further reaction with cell damaging by ROS.

Protection against acute doxorubicin-induced toxicity was confirmed on other examined parameters red blood cells (Djorjdevic-Milic et al., 2009), liver (Milic Torres V. et al. unpublished results), kidneys, lungs, and testicles, caused by administration of DOX in healthy Wistar rats (Srdjenovic et al., 2010).

Injac et al., evaluated protective effect of C₆₀(OH)₂₄ on acute tissue oxidative injury mediated by single application of doxorubicin (8 mg/kg, i.p.) on female Sprague-Dawley rats with chemically induced mammary carcinomas. In this study, ultrastructural analysis of

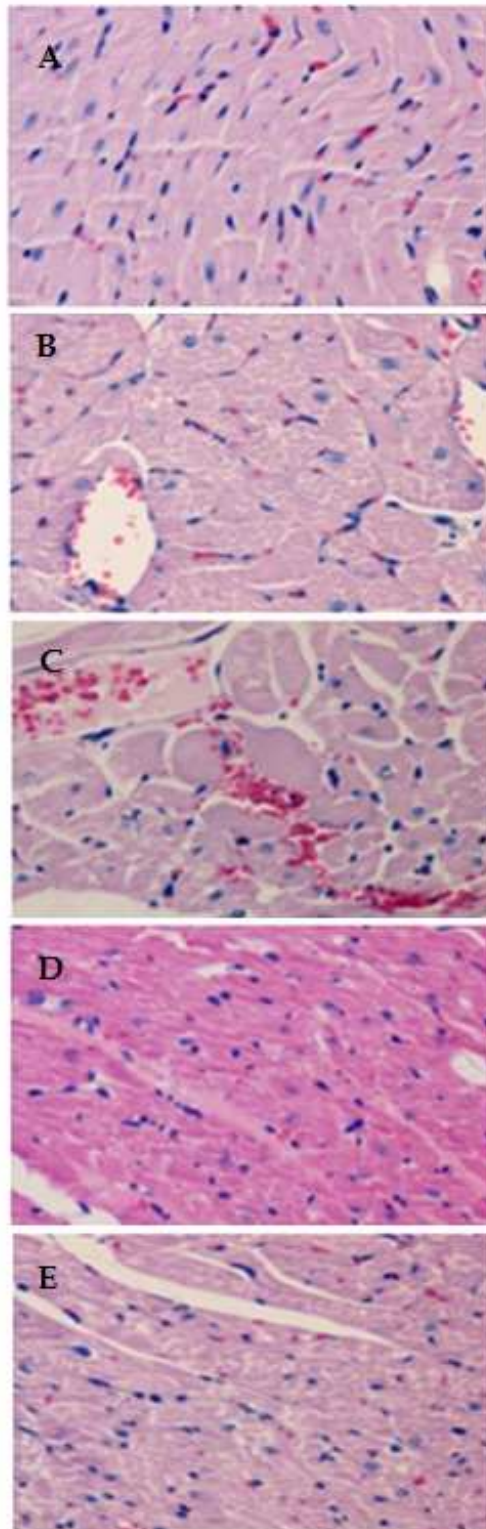


Fig. 7. Histological section of the heart (HE, 40x): A) control group - no histological lesions found; B) group treated with doxorubicin - appearance of numerous vacuoles, total degeneration of normal tissue structure C) group treated with 50mg/kg of fullereneol 30 min. prior doxorubicin - focal hemorrhage, total degeneration of myocytes ; D) group treated with 100mg/kg of fullereneol 30 min (Milic Torres et al., 2010).

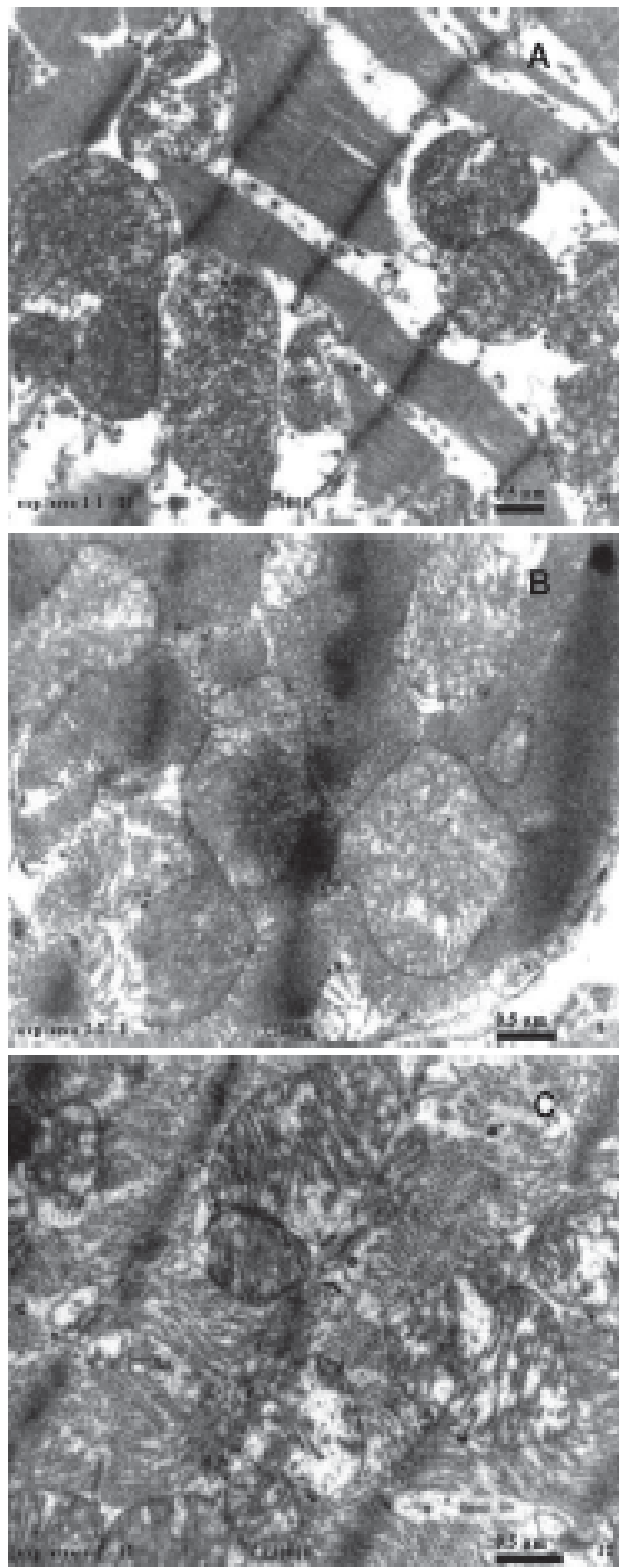


Fig. 8. Mitochondria showing (A) normal morphology, the double membrane envelope and lamellar cristae in the control rats; (B) irregular shape with lucent matrix and disorganised cristae in the Dox rats; and (C) amorphous material and rare disintegrated cristae in the Dox/Full rats (Injac, R. et al., 2008a).

ventricular tissues showed marked myocardial damage upon doxorubicin treatment. Ultrastructural analysis of the heart tissue from rats treated with doxorubicin and fullereneol indicated that hearts of the animals were protected from doxorubicin-induced subcellular damage (Figure. 8.). Again, it was confirmed oxidative nature of cardiac injury caused by anthracycline application (elevated parameters of oxidative stress - SOD, MDA, GST, GSH, GR and TAS) and antioxidative capability of fullereneol, which maintain all examined parameters at the basal level, when is applied as pretreatment to doxorubicin (Injac, R. et al., 2008a)

Protective effects of fullereneol $C_{60}(OH)_{24}$ on Wistar rats with colorectal carcinoma (chronic regimen of DOX administration - 1.5mg/kg/week, for 3 weeks)) was assessed by the same research group. They findings confirmed cardioprotective role of fullereneol, without modulation of antitumor activity of the drug. The myocardial lesions caused by doxorubicin administration were significantly reduced in animals received fullereneol. Functional and biochemical examination of the heart were in concordance with pathohistological findings. Moreover, fullereneol exhibit significantly higher degree of protection over the well known antioxidant - vitamin C, which was used as a positive control (Injac, R. et al., 2009).

In favour to $C_{60}(OH)_{24}$ cardioprotectivity are going biodistribution studies of fullerene. Ji et al, found that the high concentration of the $^{125}I-C_{60}(OH)_x$ in the heart was able to detect even three days after the administration (Ji et al., 2006)

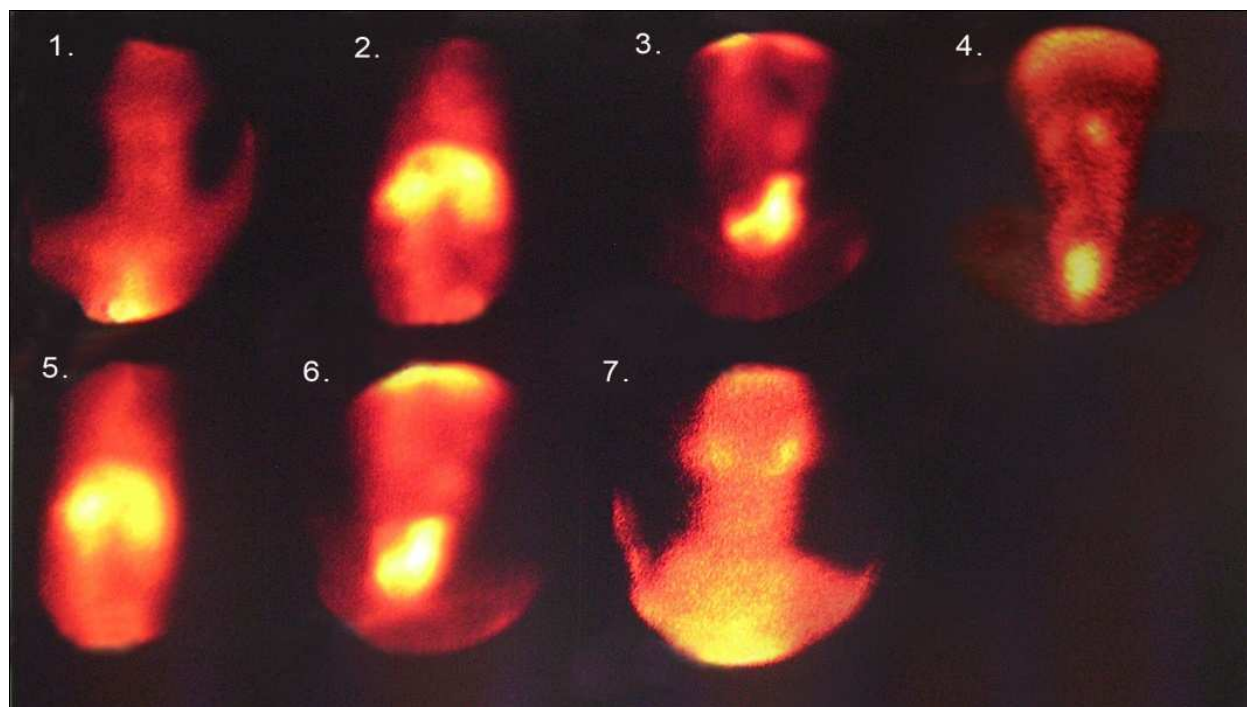


Fig. 9. Static scintigraphy analysis of dog: 1. heart activity after 1 hour; 2. liver activity after 1 hour; 3. kidney and urinary bladder activities after 24 hours; 4. kidney and urinary bladder activities after 21 hours; 5. liver, kidneys and urinary bladder activities after 24h; 6. salivary glands activity after 24 hours; 7. heart activity after 24 hours

Dynamic scintigraphy of the domestic dog, using $^{99m}TcO_4$ -tagged fullereneol ($[^{99m}Tc(CO)_3(H_2O)_3]-C_{60}(OH)_{22-24}$) discovered its presence in the heart, liver and spleen.

After administration of radiopharmaceutical, activity was first recorded on the heart, followed by liver and spleen (Figure. 9.). However, thirty minutes after, activity in all three organs was stabilized. The static scintigraphy reveals activity in the heart, liver, spleen and intestine after 1h, in salivary glands after 4 h and after 21 h the activity was detected in kidneys, urinary bladder and urinary tract. After 24 hours, the activity was detected in liver, spleen, kidneys and urinary bladder (Figure. 9.). These results suggest liver and urinary elimination mechanisms of tested xenobiotic with clearance of >24h (Djordjević et al., 2010).

Special interest for further investigation with fullereneol will be focused on its radioprotective activity in animal models, and on cardioprotective effects in doxorubicin-induced cardiotoxicity. However, main drawback for application of fullereneol as a system tissue protector is its water solubility of 0.44 mg/l, which is not satisfactory (Injac, Rade et al., 2008b). Enchasing of solubility using surfactants (Milic Torres et al., 2011) or co-solvent (Injac, Rade et al., 2008b), can facilitate fullereneol parenteral or oral application.

All these findings suggests that fullereneol $C_{60}(OH)_{24}$ have all properties of ideal cardioprotector against doxorubicin-induced cardiotoxicity - selectivity, ROS scavenger, iron chelator, low toxicity and no modulatory effect on antitumor activity of anthracyclines. Further *ex vivo* experiments as well as improvement of pharmaceutical formulations of the fullerene are strongly needed.

6. Natural products

The production of free radicals as a by product of DOX metabolism is the cardiotoxic mechanism with earliest recognition and widest attention. Therefore, searching for ideal protection against free radical injury was and still is a big scientific challenge. Application of natural antioxidants was first and logical path in this journey.

Numerous studies about protective effects of vitamin E against DOX-induced cardiotoxicity are quite controversial. *Vitamin E*: Protective effect vitamin E exerted against chronic DOX-induced toxicity on rabbit model when is administrated in high dosage as a pretreatment (170 mg/kg)(Van Vleet & Ferrans, 1980) or given in combination with vitamin A, reducing myocardial damage for 30% and maintained heart contractility (Milei et al., 1986). Berthiaume and co-workers found that alpha-tocopherol supplemented diet result in significant enrichment of cardiac mitochondrial membranes with vitamin E and diminished content of oxidized cardiac proteins associated with DOX treatment. However, vitamin E supplemented diet failed to protect against mitochondrial dysfunction and cardiac histopathology. These findings suggested that tocopherol enrichment is not sufficient to protect cardiac mitochondrial membranes from DOX induced toxicity (Berthiaume et al., 2005). In doxorubicin treated leukemic mice, free alpha tocopherol and tocopherol acetate cause potentiation of doxorubicin toxicity (Alberts et al., 1978). High dosage of vitamin E failed to protect the heart tissue of the rabbits received cumulative dose of 400 mg/sq m of DOX in chronic regimen. Notwithstanding, coadministration of vitamin E result in increasing life span (Breed et al., 1980). Vitamin E administrated in high dosage of 5000 IU/day, in chronic regimen (17 days) just cause mild amelioration of DOX-induced cardiotoxicity in miniature swine(Herman & Ferrans, 1983). Alpha-tocopherol in oral dose of 2g/m² applied daily enriches human serum with vitamin E from six to eight fold. However, coadministrated with DOX in cumulative dose level of 550 mg/m² did not offered

substantial protection against congestive heart failure which appeared in tested human individuals (Legha et al., 1982b). *Vitamin A*: Pretreatment of rats in dosage of 25 IU/kg of vitamin A, two days before single dose administration of DOX (10mg/kg *i.v.*) substantially reduced the peroxidative damage of the heart lipids and proteins, and markedly lowered serum values of lactate dehydrogenase and creatine kinase. This applied regimen sustained structural integration of the heart and prolonged life span of tested animals pointing on protective role of vitamin A in DOX-induced cardiotoxicity (Tesoriere et al., 1994). Lu and co-workers suggests strong cardioprotective activity of *beta-carotene*, when is given as a pretreatment to DOX (Lu et al., 1996). *L-carnitine* appeared to be cardioprotective in doxorubicin treated rats, due to improved cardiac energy metabolism and reduced lipid peroxidation (Luo et al., 1999). Pretreatment of isolated rat cardiac myocytes with *L-carnitine* (200 µg/ml) found to inhibit the doxorubicin induced sphingomyelin hydrolysis, ceramide generation and cell death in dose dependent manner (Andrieu-Abadie et al., 1999). Application of *alpha-lipoic* acid exerted cytoprotective activity against free radical injury induced by doxorubicin. It succeed to maintain biochemical parameters of oxidative stress of treated rats and to sustain structural integrity of the rat heart as well (Al-Majed et al., 2002; Balachandar et al., 2003). Extensive review was made about *coenzyme Q-10* in prevention of anthracycline-induced cardiotoxicity. Both, preclinical and clinical studies suggest its cardioprotective role without compromising antitumor activity of the DOX. Applied in daily dose range between 50 and 120 mg/day coenzyme Q-10 can ameliorate majority of side effects associated with anthracycline administration including heart failure (Conklin, 2005). Wistar rats were treated with doxorubicin (4mg/kg) and *lycopene* (5 mg/kg body weight a day) during seven week period. Morphologic examination revealed that doxorubicin-induced myocyte damage was significantly suppressed in rats treated with lycopene. Lycopene supplementation provided myocyte protection without preventing interstitial collagen accumulation increase, although cardiac dysfunction was not preserved (Anjos Ferreira et al., 2007). Lycopene may reduce or prevent the side effects of chemotherapy due its antioxidative and anti-inflammatory properties (Sahin et al., 2010). *Probucol*, a lipid-lowering agent with known antioxidative properties, coadministered with DOX to the male Sprague-Dawley in dosage of 10 mg/kg during four weeks with pretreatment of additional two weeks, completely abolished increase in oxidative stress, glutathione peroxidase (GSH-Px) inactivation and Mn dependent SOD downregulation during DOX cardiomyopathy. Li et al. suggested that rather oxidative stress mediated changes at the enzyme protein level playing role in toxicity of DOX than downregulation of the GSH-Px gene transcription or translation (Li, T. et al., 2000; Li, T. & Singal, 2000). *Low molecular weight heparin* (LMWH) administration to DOX-induced rats prevented the rise in serum and tissue levels of LDH, aminotransferases and ALP, while these parameters were significantly elevated in the DOX group in comparison with the control group. Cardiotoxicity indicated by rise in serum CPK in the DOX group was attenuated by LMWH treatment. LMWH decreased the cardiac lipid peroxidation induced by DOX. Histologic examination revealed that the DOX-induced deleterious changes in the heart were offset by LMWH treatment (Deepa & Varalakshmi, 2003). Chlopckikova and co-workers were tested chemoprotective effects of caffeic (CA), chlorogenic (CHA) and rosmarinic (RA) acids the toxicity of doxorubicin in neonatal rat cardiomyocytes and the iron-dependent DOX induced lipid peroxidation of heart membranes, mitochondria and microsomes. The test

compounds protected cardiomyocytes against DOX induced oxidative stress (RA > CHA > or = CA) on all monitored parameters. Substantial preservation of monolayer integrity of the cardiomyocytes by test compounds was also found microscopically. All the acids were more effective in the assays used than dexrazoxan. RA showed the most effective cytoprotectivity. All the acids significantly reduced the iron-dependent DOX induced lipid peroxidation of heart membranes, although CHA from the all tested compounds was found to be the most effective (Chlopcikova et al., 2004). Efficacy of the aqueous extract of the *Centella asiatica* was evaluated on the mitochondrial enzymes; mitochondrial antioxidant status in adriamycin induced myocardial injury. Pretreatment of the Wistar rats during three weeks with aqueous extract of *Centella asiatica* (orally 200 mg/kg body wt/ day) followed by co-treatment with DOX (2.5 mg/kg, i.p. two weeks) effectively counteracted the alterations in mitochondrial enzymes and mitochondrial defence system. In addition, transmission electron microscopy study confirms the restoration of cellular normalcy and accredits the cytoprotective role of *Centella asiatica* against adriamycin induced myocardial injury. Results of Gnanapragasam et al. suggest that the aqueous extract of *Centella asiatica* not only possesses antioxidant properties but it may also reduce the extent of mitochondrial damage (Gnanapragasam et al., 2007). Oral administration of *Aloe barbadensis* gel – aloe vera (100 and 200 mg/kg), to the albino rats during 10 days, produced a significant protection against cardiotoxicity induced by DOX (single dose 7.5 mg/kg *i.v.*). Aloe vera gel kept serum levels of LDH, CPK, cardiac lipid peroxides, tissue catalase and tissue SOD along with the blood and tissue GSH on the basal values. The results revealed that aloe vera gel exhibit a dose dependent protection against DOX induced cardiotoxicity (Kaithwas et al., 2011). Effect of methanolic extract of fruits of *Piper longum* (PLM) on the biochemical changes, tissue peroxidative damage and abnormal antioxidant levels in DOX induced cardiotoxicity in Wistar rats was investigated by Wakade and co-workers. Piper longum extract was administered to the Wistar rats in two different doses, by gastric gavage (250 mg/kg and 500 mg/kg) during three weeks followed by unidose of DOX (15 mg/kg, i.p.) at the 21st day. Activities of myocardial antioxidant enzymes (CAT, SOD, GSH-Px, GR and GSSG) were significantly lowered due to cardiotoxicity in rats administered with DOX. PLM pretreatment maintain of these endogenous antioxidants on the level of control. Structural examination of the heart revealed degenerative changes and cellular infiltrations in rats administered with DOX and pretreatment with PLM reduced the intensity of such lesions. The results indicate that PLM administration offers significant protection against DOX induced oxidative stress and reduces the cardiotoxicity of the administrated antineoplastic drug (Wakade et al., 2008). Flavonoid scavenging activity of *propolis* has been investigated against oxidative injury of the heart, induced by single dose administration of DOX (20 mg/kg, *i.p.*). Pretreatment of rats with propolis extract, given per os (100 mg/kg/day) during four days prior to DXR injection, substantially reduced the peroxidative damage of the heart mitochondria. It was evident significant reducing both mitochondrial MDA formation and production of superoxide anion. These data are demonstrated potent role of propolis extract counteracting doxorubicin caused cardiotoxicity (Alyane et al., 2008). Strong cardioprotection based on ROS scavenging activity against acute DOX induced toxicity was exhibited by: *hesperetin* – hypocholesterolemic citrus flavonoid (50 and 100 mg/kg) (Trivedi et al., 2011); *melatonin* (2x 5mg/kg) (Aydemir et al., 2010); *resveratrol* (10 mg/kg) (Danz et al., 2009; Olukman et al., 2009; Tatlidede et al., 2009); *procyanidins* from grape seeds (15

mg/kg)(Li, W. et al., 2009); *silymarin*, flavonolignans extracted from *Silybum marianum* (50 mg/kg) (El-Shitany et al., 2008) and *salvianolic acid* from *Salvia miltiorrhiza* (3 x 40mg/kg before DOX)(Jiang et al., 2008).

It is obvious that diverse natural products have powerful ability to counteract the toxicity of doxorubicin and other anthracycline antibiotics. Unfortunately, the results from the studies conducted so far, are from pre clinical phase, rarely some of them reach clinical trials, and none is defined as a commercial protector. Use of natural antioxidants, against DOX induced cardiotoxicity, according to our knowledge, are recommended by a physician as a supplement to the treatment protocols in oncology. There are no scientific and clear evidences of their beneficial effects in large cohorts.

7. Conclusion

Cardiac complications induced by doxorubicin therapy are of considerable importance today as when was appeared 30 years ago. Furthermore, the number of the patients surviving cancer and chemotherapy is bigger nowadays and appearance of subclinical cardiac dysfunction is even more frequent. Mechanisms underlying cardiotoxicity of DOX are complex. In spite of multitude hypotheses involving gene expression changes, activation of ubiquitin-proteasome system, cell death as well as innate immunity activation, oxidative injury of the heart and alteration in iron homeostasis most likely to have primary role in cardiotoxicity developed by doxorubicin. No single drug will be able to prevent cardiotoxicity. Therefore, more clinical studies are needed to elucidate the mechanism and develop strategies in prevention against DOX-induced cardiotoxicity.

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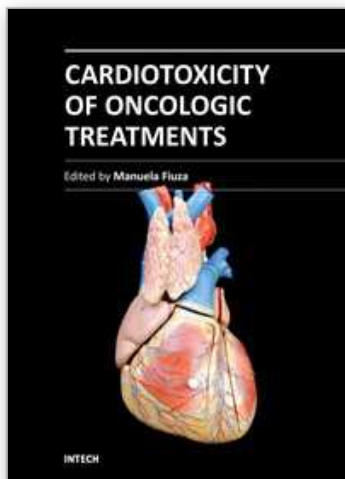
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The possibility of getting a cardiovascular disease or cancer increases with advancing age. At the same time, relevant improvements in cancer therapy have resulted in the improvement of quality of life and the increasement of the survival rate of such patients. As a result we have larger number of patients that experience the cardiac side effects of chemotherapy. The extent of cardiotoxicity is variable, depending on the type of drug used, combination with other drugs, prior mediastinal radiotherapy and the presence of cardiovascular risk factors or history of heart disease. Early detection of the patients proneness for developing cardiotoxicity is the key issue to decrease morbidity and mortality. It also facilitates more tailored therapeutic interventions. Therefore, the collaboration and interaction of cardiology and oncology may contribute to reducing the cardiovascular adverse effects and improving the results in the treatment of patients with cancer.

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Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
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InTech China

Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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