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Research Article

TRANSMISSION ELECTRON MICROSCOPE STUDY OF THEAMELIORATING EFFECT OF ARONIA MELANOCARPA ON DOXORUBICIN-INDUCED CARDIOTOXICITY

V. Pavlova[1]*, B. Alexieva[1], I. Sainova[1], TZ. Markova[2], E. Nikolova[1], S. Kuzmanova[3], M. Krachanova[4] and P. Denev[4]

[1]Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Acad. G. Bonchev str., bl. 25, Sofia 1113, Bulgaria.
[2] Department of Pharmacology and Toxicology in MedicalUniversity, 15 Acad. I. Geshovblvd, Sofia 1431, Bulgaria.
[3]Department of Preclinical and Clinical Pharmacology and Biochemistry, MedicalUniversity, Varna, Bulgaria.
[4]Laboratory of Biologically Active Substances, Institute of Organic Chemistry with Centre of Phytochemistry, Plovdiv, Bulgaria.

ABSTRACT

Doxorubicin (DOX) is one of the most effectiveand commonly used antitumor drugswith high antineoplastic activity to a broad spectrum of cancers.The therapeutic value of DOX, however, is limited by its dose dependent cardiotoxicity. The free radical-induced cardiac oxidative stress and reactive oxygen species –caused damage is considered as one of the underlying mechanisms of DOX-induced cardiotoxicity. Aronia melanocarpa (Black chokeberry) fruits are rich sources of phenolic substances with one of the most potent antioxidant activities among plant species. Aronia melanocarpa polyphenols promote the restoration of the main intracellular antioxidant glutathione (GSH) in the state of oxidative stress, which is crucial for counteracting the free radical-induced intracellular injury. The aim of our study was to investigate the possible protective effect of Aronia Melanocarpa fruit juice (AMFJ) against DOX-induced cardiotoxicity and oxidative stress in an *in vivo* mouse model. The administration of DOX (20 mg/kg i. p.) to Balb/c experimental mice caused significant decrease of tissue glutathione level in heart samples, as well as marked ultrastructural changes with different degree of damage of cardiomyocytes, examined by electrone microscope. These biochemical and histological alterations were effectively attenuated on pretreatment with AMFJ. We concluded that AMFJ had ameliorating effect on DOX-induced cardiotoxicity and the mechanism of this cytoprotective action is related to the enhancement of GSH antioxidant pool and reduction of cellular oxidative stress induced by DOX treatment.

Keywords: Antioxidants, Black chokeberry, Cardiotoxicity, Chemotherapy

INTRODUCTION

The aim of chemotherapy is to slow or to prevent the progression of primary or secondary malignancies by systemic use of drugs with cytotoxic effect against highly proliferating cells. Unfortunately, these drugs target not only cancerous, but also other cells, thus causing damages to normal tissues.

Doxorubicin belongs to the anthracyclines family andit was isolated from a pigment of *Streptomyces peucetius* and introduced in 1969 for cancer treatment. Since then DOX remains one of the most effective and widely used anti-tumor drugs ever developed, with high anti-neoplastic activity to breast cancer, aggressive lymphomas, childhood solid tumors and soft tissue sarcomas (Minotti et al., 2004; Quiles et al., 2006, Volkova and Russell, 2011). However, DOXhas shown adverse side effects like hematopoietic suppression, nausea, vomiting and alopecia, but the most feared adverse reaction is its cumulative and dose-dependent cardiac toxicity. The clinical manifestation of cardiotoxicity can range from sub-clinical abnormalities as asymptomatic electrocardiography changes to life-threatening and sometimes fatal events as congestive heart failure (Chen et al., 2007, Quiles et al, 2006).Although intensive investigations on DOX-induced cardiotoxicity have been continued for decades, the precise mechanisms have not been completely elucidated. It has been suggested that one of the underlying molecular mechanism responsible for DOX toxicity is the formation of reactive oxygen and nitrogen species (ROS and RNS) (Kim et al., 2006), followed by lipid peroxidation and decreased glutathione (GSH) levels (Alfonso et al., 2001). When the formation of ROSand RNS exceeds cellular adaptive and repair capacities, a condition that is referred to as oxidative stress occurs, in which biological molecules such as nucleic acids, proteins and membrane phospholipids become damaged through oxidative reactions.

GSHisathiol-containingtripeptide (L-γglutamil-L-cysteinyl-glycine), whichisubiquitousinthecells. It is the primary intracellular antioxidant that neutralizes oxidative stress, detoxifies toxins and scavenges ROS formed during normal metabolic process or as a result of trauma, infection or medication. This ability makes GSH central to defense mechanisms against intra and extra-cellular oxidative stress and its availability is crucial for antioxidant protection in a biological system. GSH deficit disrupts redox-status and upsets the physiological cellular balance between pro-oxidants and antioxidants. Lowered cellular GSH is observed in different pathological conditions and its modulation can represent a supportive measure in achieving a therapeutic goal (Wu et al, 2004).

Recently much attention has been focused on the protective effects of antioxidants and naturally occurring substances against DOX-induced cardiotoxicity (Principal et al. 2010; Chularojmontri et al., 2013; Stoner et al., 2008; Wang and Stoner, 2008). In XX century, the medicinal plant *Aronia melanocarpa* has become popular in many countries not only with its valuable food qualities, but also as a prophylactic and therapeutic supplement (Domarew et al., 2002; Hovmaln Persson et al., 2004; Kokotkiewicz et al., 2010). As a rich source of polyphenols and anthocyanins, the extract of this plant has been proved to have anti-hypertensive, anti-atherosclerotic, anti-proliferative, and chemo-protective properties (Denev et al., 2012; Domarew et al., 2002; Kähkönen et al., 1999; Kong et al., 2003; Wang and Stoner, 2008; Zdunczyk et al., 2002; Zhao et al., 2004). In the study of Rugina et al. (2011), the protective action of chokeberry extract against oxidative stress induced by high doses of glucose in pancreatic cells was evaluated. The results indicated a strong scavenging effect of chokeberry anthocyanins on the intracellular ROS species and an ability to restore dose-dependently the strong decrease of GSH. Another research group, Zhu et al., 2012, examinedthe mechanism of the *Aronia* anthocyanin-mediated increase of GSH synthesis and protection of hepatocytes against ROS–induced injury.

In the current study, we hypothesized that AMFJ is capable of stimulating GSH synthesis, promoting drug detoxification and acting directly as a scavenger of free radicals, derived from DOX treatment of experimental mice.

MATERIALS AND METHODS

Experimental animals and experimental protocol

Male and female Balb/c mice, aged 3 months and weighing 20-25g, came from Slivnicaanimal breeding house, Sofia. They were randomized into 4 groups of 6 animals: treated with AMFJ (*Aronia*-group); treated with Doxorubicin (DOX group), treated with both AMFJand Doxorubicin (*Aronia* + DOX group), and untreated healthy controls (Control group). AMFJ was kindly provided by S. Kuzmanova from the Department of Preclinical and Clinical Pharmacology and Biochemistry in Medical University in Varna and it contains 5461 mg/l polyphenols, 3122.5 mg/l pro-anthocyanidins and 221.4 mg/l cyanidins. All animals were fed the standard chow diet.

Aronia supplementation was made for 28 consecutive days and the fruit juice was given to mice from *Aronia* and (*Aronia* + DOX) groups as 20% water solution instead of water. Doxorubicin hydrochloride (Sigma-Aldrich), was freshly prepared in saline solution and given to animals as a single intra-peritoneal (i. p.) injection of 20 mg/kg b. wt. to DOX and (DOX + *Aronia*) groups on the 26-*th*day of the beginning of the experiment. Mice from untreated controls and *Aronia* group were injected with saline intra-peritoneally on the same day. After 28 days of *Aronia* pre-treatment and 2 days of DOX injection, all mice were sacrificed. Heart samples were taken and proceeded separately for routine ultrastructural examination by transmissionelectron microscope and for biochemical measurement of reduced GSH. All animal procedures were performed in accordance with Animal Ethics Committee.

Assessment of biochemical and morphological characteristics

Tissue samples of hearts from the experimental and control mice were isolated, and after mechanical homogenization were treated with 10% trichloroacetic acid (Cl_3CCOOH), 0.48M solution of K_3PO_4 and centrifuged at 3000 x for 10 min. The supernatants were used to determine the reduced glutathione by a spectrophotometric method (Ellman, 1959), and absorbances were measured at 412 nm (SPEKOL 1500, Analytik Jena). The level of GSH was defined from the standard curve with commercially available GSH (Sigma-Aldrich) and the results were expressed as micromole per 1 gram wet tissue (µM/g wet tissue).

Hearts for ultrastructural analysis were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), post-fixed in 1% osmium tetroxide (OsO_4), dehydrated through an ascending ethanol series and embedded in Durcopan resin. Ultrathin sections of the left ventricular wall were examined under transmission electron microscope JEM-1200 Ex/ASID, JEOL.

Statistical analysis

All data for GSH level in the heart samples are expressed as mean values±standard deviations (SD) for six animals per group. The significance of differences in GSH content in the cardiac tissue of control and experimental mice was evaluated using Student's t test. The level $P \leq 0.05$ was used as the criterion for significance.

RESULTS

GSH level in cardiomyocytes

The effects of DOX and DOX combined with AMFJ supplementation on cardiac GSH content are shown in Fig 1. DOX treatment caused significant reduction (about 35%) in GSH cardiac content, compared to Controls (0.62 ± 0.25 *vs.*0.97 ± 16µmol/g wet tissue, $P \leq 0.05$). AMFJ supplementation, however, restored in part GSH level but it did not reach that of the control group. Animals from DOX+Aronia group showed only about 6% reduction of cardiac GSH content compared to Controls (0.92 ± 0.11 *vs.*0.97 ± 16µmol/g wet tissue, $P \leq 0.05$). There was not a significant difference between GSH levels in Aronia group and Control group of mice.

Ultrastructural analysis

Results from electrone microscope study are shown in Fig. 2. Heart samples from Conrol mice showed normal morphology-regular arrangement of cardiomyocytes with abundant elongated or spherical in shape mitochondria arranged regularly in rows in-between the myofibrils. Each mitochondrion contained closely packed well visible cristae (Fig 2 A and B). Electronic microscope photomicrographs from heart samples of mice from Aronia group showed similar appearance to those of the Control group (data not shown). Ultra structural pathology, however, was apparent in the myocardium of DOX-treated mice: loss of cross striations and abundant cytoplasmic vacuolization, as well as disorganization and fragmentation of myofibrils (Fig. 2 C and D). In addition, there were wide spaces which reflect the presence of edema in-between the myofibrils. The mitochondria were with irregular arrangement, variable in shape and size, with deformation and disruption of mitochondrial membrane, cristae distortion and disappearance. In DOX-treated and AMFJ supplemented mice marked reduction in the ultrastructural pathological changes induced by DOX were observed. The organization of cardiac myofibrils was preserved to a great extent and some minimal edematous spaces and vacuolization in the intramyofibrillary areas were seen, but no mitochondrial damage was observed (Fig. 2 E and F).

DISCUSSION

DOX is effective and broadly used chemotherapeutic drug which has a proven efficacy in a number of solid tumors, acute leukemia and lymphoma. Unfortunately treatment with DOX is limited because of its cardiotoxicity. Since free radicals and oxidative

stress are apparently involved in the mechanisms of DOX-induced cardiac toxicity, several investigators have reported with varying degrees of success the protective activity of naturally occurring antioxidants against DOX-induced cardiotoxicity. In our study we chose chokeberry fruit juice, which possesses one of the highest antioxidant activities among plant species. The available data from *in vitro* and *in vivo* studies on chokeberry antioxidant effect showed that it extends beyond radical scavenging and suppression of ROS and RNS formation. It was recently confirmed that Aronia melanocarpa polyphenols promote the restoration of the main intracellular antioxidant GSH in the state of oxidative stress, which is crucial for counteracting the free radical-induced intracellular injury. It is evident that the most cellular damages occur after the depletion of GSH, which sets out the onset of uncontrolled oxidative injury. It has been shown that reduction of GSH pool impairs the cellular capacity in antioxidant defence system and likewise, increased GSH pool is associated with cytoprotection against oxidative damage. Dietary GSH sources are few and its excess does not increase the maximal hepatic GSH amount beyond the normal physiological level, due to the feedback regulation of GSH level.

Cardiac myocytes contain the highest volume density of mitochondria in the body, occupying about 40% of the total intracellular volume. DOX has a high affinity for cardiolipin, a negatively charged phospholipid abundant in the mitochondrial inner membrane, leading to mitochondrial accumulation of DOX. This leads to a high redox reactivity and severe mitochondrial damage in cardiomyocytes (Wallace, 2003). It is of value to remember that heart tissue is very sensitive to free radical injury because of its highly oxidative metabolism and the lower amount of endogenous antioxidants in this organ.

In our experiment, DOX administration caused ROS generation, and oxidative stress, which reflects in GSH depletion due to consumption of intracellular GSH after the influx of DOX and its toxic metabolites. Electron microscopy revealed severe ultrastructural pathology in cardiomyocytes. The oxidant burden, however, was effectively attenuated after AMFJ pre-treatment and the partly restoration of GSH content in cardiomyocytes reflects the marked reduction in the ultrastructural pathological changes induced by DOX, as evidenced in the representative photomicrographs of the heart samples of (DOX+Aronia) group of mice.

Improvement of GSH-associated metabolism is a major mechanism for cellular protection against agents which generate oxidative stress. It is becoming increasingly apparent that the GSH tripeptide is central to a complex diverse detoxification system, where there is substantial inter-dependence between separate components. Glutathione participates in detoxification at several different levels, and may scavenge free radicals, reduce peroxides or be conjugated with electrophilic compounds. Thus, glutathione provides the cell with multiple defenses not only against free radicals, but also against their toxic products.

CONCLUSION

ROS and RNS play an essential role in the toxicity associated with DOX treatment. Our study demonstrated that AMFJ had ameliorating effect on DOX-induced cardiotoxicity of Balb/c mice via mechanisms related to the reduction of cellular oxidative stress and enhancement of GSH antioxidant pool. Further studies on glutathione-dependent enzymes representing coordinately regulated defense mechanisms against oxidative stress should be necessary. Also, investigation on the influence of the separate components of *Aronia melanocarpa* (polyphenols and anthocyanins) on the levels of intracellular GSH should be provided.

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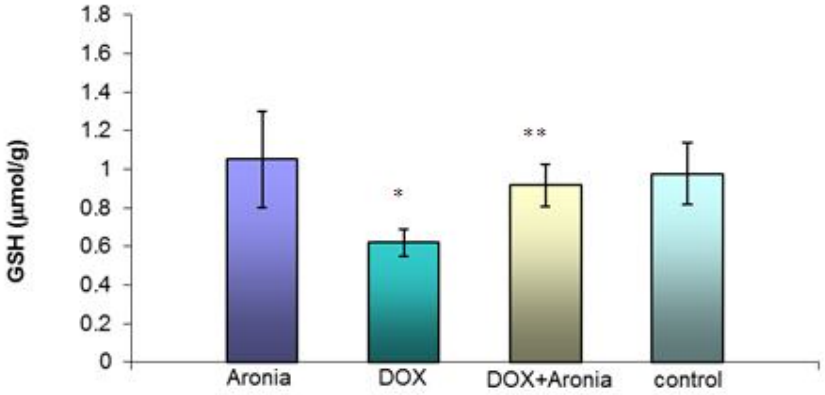

Fig. 1: GSH content (µmol/g wet tissue) in heart samples of experimental and control Balb/c mice.

* Significantly different from Control group of mice (p≤0.001)
**Significantly different from DOX-treated group of mice (p≤0.001)
Values are sxpressed as mean±SD, n=6

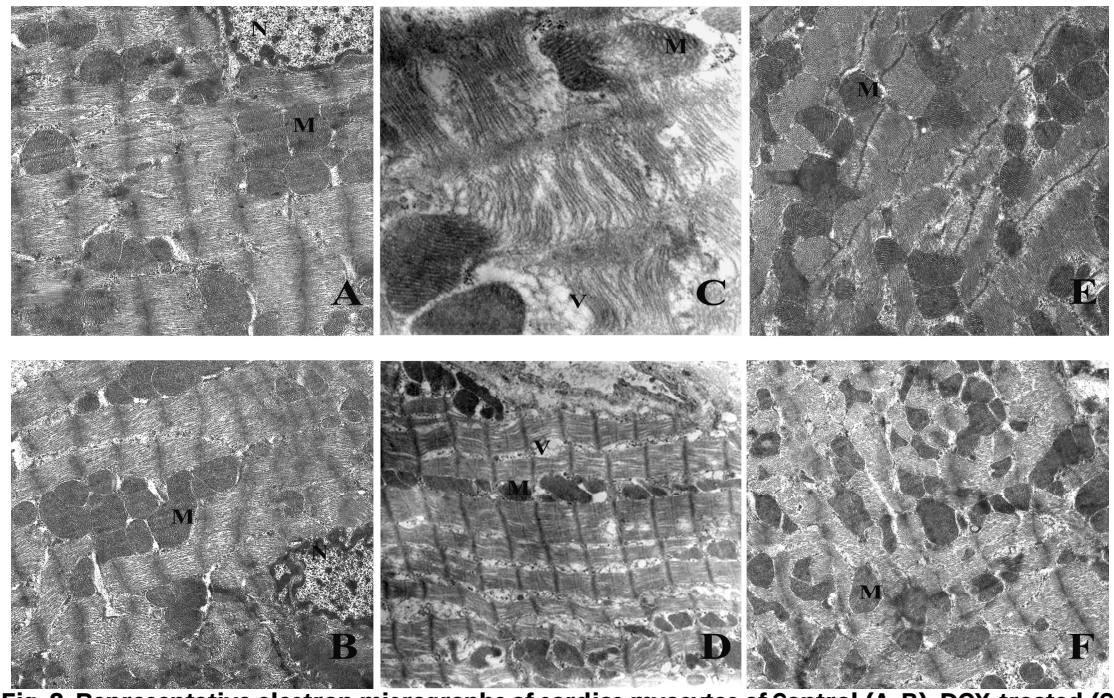

Fig. 2: Representative electron micrographs of cardiac myocytes of Control (A, B), DOX-treated (C, D) and DOX +Aronia-treated (E, F) group of mice. Original magnification : A - 10 000x; B, E and F - 15 000x; C - 25 000x; D - 8 000x. M - mitochondrion; V- cytoplasmic vacuolization; N - nucleus

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