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ANTIOXIDANT EFFECT OF *ARONIA MELANOCARPA* EXTRACT AFTER DOXORUBICIN TREATMENT

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Abstract

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Doxorubicin is one of the most effective chemotherapeutic agents in current use. However, its therapeutic value is limited by its toxicity to non-targeted tissues. Although the mechanisms of this toxicity are not fully elucidated, oxidative stress appears to be involved. *Aronia melanocarpa* (*Black chokeberry*) has shown a high content of polyphenol compounds and possesses one of the highest antioxidant activities among fruits. We investigated the possible protective effect of total extract from this medicinal plant against Doxorubicin-induced toxicity and oxidative stress. The administration of Doxorubicin (20 mg/kg i. p.) to Balb/c experimental mice caused significant decrease of tissue glutathione level in liver, heart and small intestine, as well as marked histo-pathological changes, examined by light microscopy. These biochemical and histological alterations were effectively attenuated on pretreatment with *Aronia melanocarpa* total extract. We concluded that the protective action of *Aronia melanocarpa* total extract is due to the enhancement of tissue glutathione level, which might have important cytoprotective effects on oxidative stress induced by Doxorubicin treatment.

Key words: Chemotherapy, Antioxidants, *Aronia melanocarpa* total extract, Glutathione, Cardiotoxicity
Abbreviations: DOX, doxorubicin; ROS, reactive oxygen species; RNS, reactive nitrogen species; GSH, glutathione

Introduction

Doxorubicin (DOX), along with daunorubicin, idarubicin and epirubicin, belongs to anthracyclines family. DOX was isolated from a pigment of *Streptomyces peucetius* and it was introduced in 1969 for cancer treatment. Since then DOX remains one of the most effective and widely used chemotherapeutic 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). However, DOX use in chemotherapy has been limited due to its diverse toxicities. It has been suggested that one of the molecular mechanisms responsible for DOX toxicity is the formation of reactive oxygen and nitrogen species (ROS and RNS) (Kim et al., 2006), lipid peroxidation and decreased glutathione (GSH) levels (Alfonso et al., 2001). When the formation of ROS 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.

GSH is a thiol-containing tripeptide (L-γglutamil-L-cysteinyl-glycine), which is ubiquitous in the cells. 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

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stress. The activity of hydrosulfide group determines the biological significance and activity of GSH in antioxidant and detoxifying reactions (Meister, 1983). This substance is responsible for keeping proper thiol-disulfide balance and related redox-potential in the cells. Moreover, the nucleophilic glutathione–SH group enters reactions with electrophilic substances, either endogenous or exogenous (xenobiotics, including drugs), yielding glutathione S-conjugates, (i. e GSH thioeters), which are then transformed to mercapturic acids and excreted (Meister, 1983; Hayes and McLellan, 1999). Thus, the availability of GSH is crucial for antioxidant defense 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 (inflammations, Parkinson's disease, AIDS, diabetes and others), and GSH modulation can thus represent a supportive measure to achieve 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, medicinal plant *Aronia melanocarpa* has become popular in many countries all over the world not only with its valuable food qualities, but also as a therapeutic and prophylactic 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 chemoprotective 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), proved the 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 *Aronia melanocarpa* total extract 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 20–25 g, came from Slivnica animal breeding house, Sofia. They were randomized into 4 groups of 6 animals: treated with *Aronia*-extract (*Aronia*-group); treated with Doxorubicin (DOX group), treated with both *Aronia*-extract and Doxorubicin (*Aronia* + DOX group), and untreated healthy controls (Control group). *Aronia* total extract 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 total extract 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 24-rd 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 4 days of DOX injection, all mice were sacrificed. Heart, small intestine and liver samples were taken and preceded separately for routine histological examination by light microscopy 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 liver, heart and small intestine 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 absorbance 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 are expressed as micromole per 1 gram wet tissue (μM/g wet tissue).

For histological examination, tissue samples from the same organs were fixed in 10% neutral phosphate buffered formalin, dehydrated in ethanol series, embedded in Tissue-Tek embedding medium (Sakura Finetek, USA), cut in 5 μm sections and stained with Hematoxilline & Eosin. Light microscopy examination was performed under Light microscope DM 5000B, supplied with Camera Leica DFC 420C.

Results and Discussion

The effect of *Aronia* supplementation on GSH levels of *Aronia*, DOX, (DOX+*Aronia*) and Control group in different organs of Balb/c mice are summarized in Figure 1.

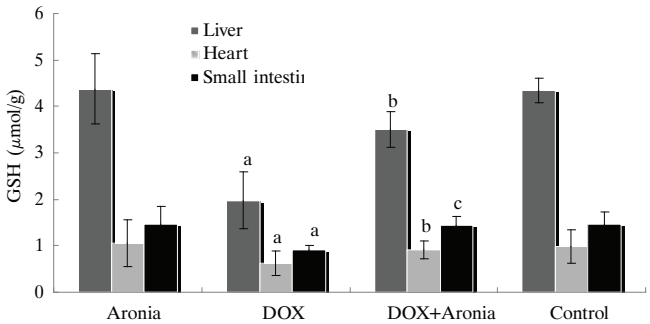

Fig. 1. GSH levels [μM/g wet tissue] in liver, heart and small intestine of Aronia supplemented, DOX-treated, DOX+ Aronia treated and Control group of mice.
***a*: Significantly different from control group ($p < 0.001$);**
***b*: Significantly different from DOX-treated group ($p < 0.001$); *c*: significantly different from DOX-treated group ($p < 0.05$)**
Values are expressed as mean ± S.D., n = 6

DOX treatment caused significant reduction in GSH content, compared to controls in all investigated organs. *Aronia* pretreatment, however, restored in part GSH level, but it did not reach those of the control group. There was not a statistically significant difference in GSH content between *Aronia* group and Control group.

The results from the light microscopy examinations are shown in Figure 2.

Photomicrographs of the heart of Control mice revealed normal myofibrilar structure with striations and branched appearance (2A). Heart of DOX treated mice, however, showed irregular, destructed and fragmented muscle fibres with cytoplasmic vacuolization (2B). Photomicrographs of animals pre-treated with *Aronia* showed better preserved appearance with nearly normal structure of the cardiac myocytes and slightly pronounced vascular dilatation (2C).

In the small intestine of mice DOX administration induced focal areas of epithelial cell layer denudation and visible shortening of the intestinal villi. *Aronia* pre-treatment considerably ameliorated this toxic effect of DOX (data not shown). No pathological changes however were observed in the liver of experimental mice after a single i.p. DOX injection.

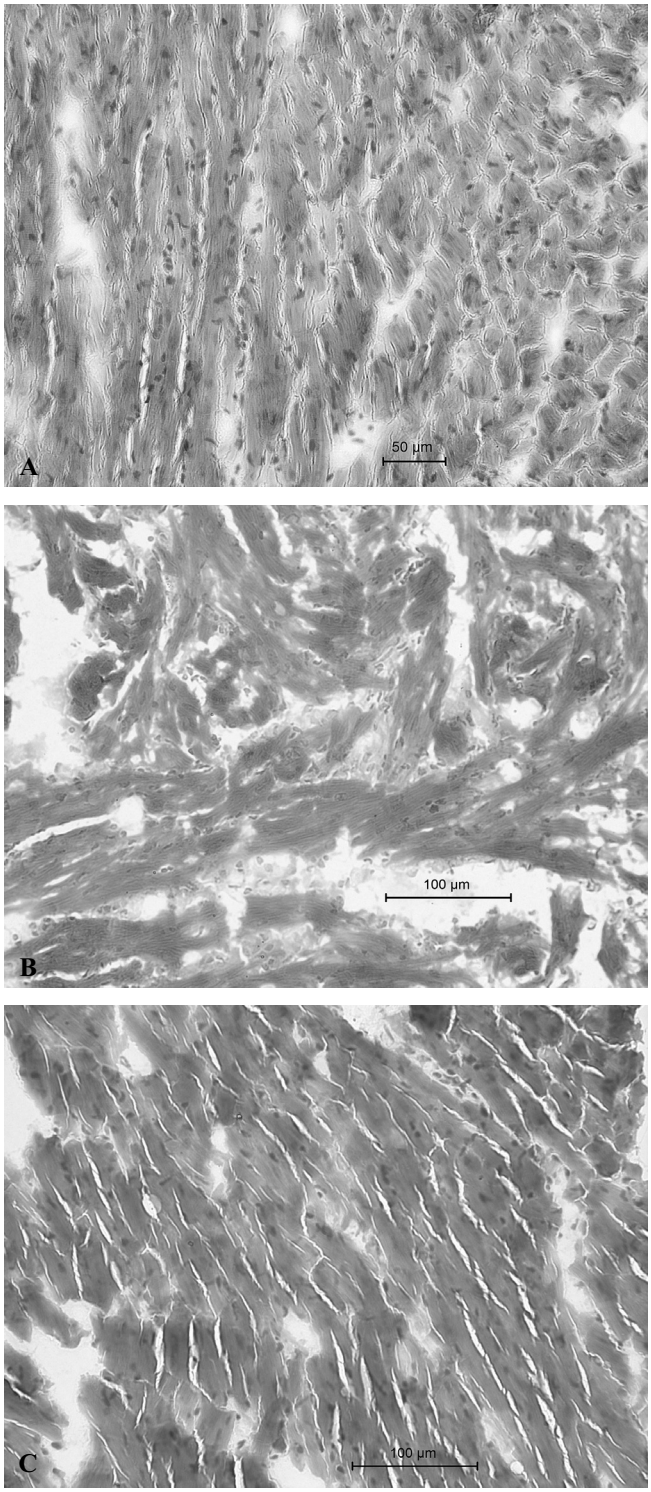

Fig. 2. Representative light micrographs of cardiac myocytes of Control (2*A*), DOX-treated (2*B*), and DOX+Aronia treated (2*C*) group of mice.(H&E)

It is evident that most cellular damage occurs 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. The liver, as a key organ for xenobiotic detoxification and elimination, is the major site of GSH synthesis (Kaplowitz et al., 1985). Almost 95% of GSH synthesized in the liver is released in the blood stream, which supplies the extra-hepatic tissues and bile. The latter is the main source for the intestinal mucosa, where its concentration is relatively high. GSH content is not as abundant in the heart as it is in the liver, which is reflected by the greater resistance of liver to DOX-induced toxicity from free radicals, and this explains the lack of histopathological changes in this organ after a single DOX administration. It is of value to remember that heart tissue is very sensitive to free radical injury not only because of the lower amount of GSH, but also due to its highly oxidative metabolism. 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. This oxidant burden however was effectively attenuated after *Aronia* extract pre-treatment as evidenced by the light microscopy examination.

Improvement of Glutathione-associated metabolism is a major mechanism for cellular protection against agents which generate oxidative stress. It is becoming increasingly apparent that the glutathione tripeptide is central to a complex multifaceted detoxification system, where there is substantial inter-dependence between separate component members. 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 ROS, but also against their toxic products.

Conclusion

ROS play an essential role in the toxicity associated with DOX treatment. Our study demonstrated that *Aronia melanocarpa* total extract had ameliorating effect on DOX-induced cardiotoxicity and to a lesser extent on small intestinal injury 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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