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ORIGINAL ARTICLE

Antioxidant Influence of *Aronia melanocarpa* Extract after Doxorubicin Treatment *in Vitro* and *in Vivo*

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ABSTRACT

Levels of the intracellular reduced Glutathione (GSH), proved as a result mainly of the action of medical plant Aronia melanocarpa (Black chokeberry) in vitro and in vivo, were measured. is one of the most effective for current use. Although the mechanisms of the toxicity of chemotherapeutic agent Doxorubicin (DOX) are not fully cleared, oxidative stress appears to be involved. Aronia melanocarpa has been shown to possess one of the highest antioxidant activities among fruits. In this connection, the current study was directed to investigation on the possible protective effect of the total extract from this medicinal plant against DOX-induced toxicity and oxidative stress both in vitro and in vivo. Despite of the established decrease of the reduced GSH levels in normal 3T3 mouse embryonic fibroblasts, malignant cells and mixed cultures in the presence of Doxorubicin, certain regeneration in them was noticed in the presence of Aronia-extract. 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. In all cases, the observed biochemical alterations were attenuated in pre-treatment with Aronia melanocarpa total extract. We concluded that the protective action of this plant extract is due to the enhancement of intracellular GSH level, which might have important cell-protective effects on Doxorubicin-induced oxidative stress. Further in vitro- and in vivo-studies on the influence of separate plant components on the levels of intracellular GSH, but also on GSH-dependent enzymes representing coordinately regulated defense mechanisms against oxidative changes, should be made.

Keywords: *Reduced glutathione (GSH), Aronia melanocarpa total extract, Doxorubicin, Oxidative stress, Antioxidant action*

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INTRODUCTION

Glutathione is a thiol-containing tripeptide (L-γglutamil-L-cysteinyl-glycine), which is ubiquitous in the cells. The activity of hydrosulfide group determines the biological significance and activity of GSH in antioxidant and detoxifying reactions [1]. 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 [2]. Thus, the availability of GSH is crucial for antioxidant defense in a biological system. GSH deficit disrupts the redox-status and upsets the physiological cellular balance between pro-oxidants and antioxidants [3]. Lowered cellular GSH is observed in different pathological conditions (pre-malignancies and malignancies, inflammations, Parkinson's disease, AIDS, diabetes and others). Thus, GSH modulation could represent a supportive measure to achieve a therapeutic goal.

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 [4]. 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) [5], lipid peroxidation and decreased glutathione (GSH) levels [6]. 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. Recently much attention has been focused on the protective effects of antioxidants and naturally occurring substances against DOX-induced cardiotoxicity [7-9].

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 [10-12]. As a rich source of polyphenols and anthocyanins, containing in it, the extract of this plant has been applied as a natural anti-hypertensive and anti-atherosclerotic drug [10], but also as anti-cancer, anti-oxidant and chemo-protective agent [9, 13-15]. In a recent study, protective action of chokeberry extract against oxidative stress induced by high doses of glucose in pancreatic cells has been shown [16]. These 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 the levels of the intracellular reduced glutathione (GSH). According another message, anthocyanin-mediated increase of GSH synthesis and protection of hepatocytes against ROS –induced injury has been proposed [17].

In the current study, we hypothesized that *Aronia melanocarpa* total extract is connected with investigation on some of the mechanisms of the influence of the *Aronia*-extract after Doxorubicin treatment, both *in vitro* and *in vivo*.

MATERIALS AND METODS

Normal fibroblasts from embryonic mouse Balb/c 3T3 line, malignant mouse myeloma cells, as well as mixed cultures from both cell types, were prepared. All cell cultures (1 x 10^6 cells/ml), were incubated at 37^0C in incubator with 5% CO_2 and 95% air humidification, in RPMI 1640, Dulbecco's Modified Minimal Essential Medium (DMEM) or a mixture of both media (1:1), supplemented with 10% Fetal Calf Serum (FCS), 100 UI/ml Penicillin, 0.25 mg/ml Streptomycin and 0.25 mg/ml Amphotericin-B, in 24-well plaques. In separate sub-populations from each of both cell types used, as well as in mixed cultures of them, were added total *Aronia melanocarpa* plant; 0.05M solution of Doxorubicin in distilled water, as well as from both tested substances, but respective untreated controls were also prepared. All cell cultures were observed by inverted light microscope, supplied with mega-pixel CCD-camera. Cells from all groups, treated with both substances, with each one of them, as well as the respective untreated controls, treated with 10% trichloroacetic acid (Cl_3CCOOH) and 0.48M solution of K_3PO_4. GSH levels from all of them were determined by a spectrophotometric method and absorbance were measured at 412nm [18]. The levels of GSH were defined from the standard curve with commercially available GSH and the results are expressed as milimole per 1 ml cell suspension (mM/ml cell suspension).

Male and female Balb/c mice, aged 3 months and weighing 20-25 g, came from Slivnica animal breeding house, Sofia. All animal procedures were performed in accordance with Animal Ethics Committee. They were separated into 4 groups of 6 animals: treated with *Aronia*-extract (*Aronia*-group); with Doxorubicin (DOX group), with both *Aronia*-extract and Doxorubicin (*Aronia* + DOX), as well as untreated healthy controls (Control group), respectively. The used *Aronia* total extract contains 5461 mg/l polyphenols, 3122.5 mg/l pro-anthocyanidins and 221.4 mg/l Cyanidins. *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 PBS and given to animals as intra-peritoneal (i. p.) injection of 20 mg/kg/body weight to DOX and (DOX+*Aronia*) groups on the 24-rd day of the beginning of the experiment. Mice from the untreated Control and *Aronia* groups were injected with PBS i. p. 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 proceeded separately for biochemical measurement of reduced GSH. Tissue samples of the three anatomic organs tested 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 minutes. The supernatants were used to determine the GSH levels by a spectrophotometric method [18], and the absorption was measured at 412nm (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).

RESULTS AND DISCUSSION

According to the results obtained, the levels of GSH in all normal embryonic fibroblasts, malignant cells and mixed cultures in cultivation with the chemotherapeutic drug alone were decreased **(Fig. 1)**. Restoration in these levels in the presence of *Aronia*-extract was observed in all cases, both in the presence and absence of the chemotherapeutic drug. Similar effects have been observed in investigation of other plant extracts or their components [9, 15, 19-22]. On the other hand, analogical protective action by natural plant antioxidants has been established on the toxicity, induced by Doxorubicin [23], as well as by other chemical drugs [24-26].

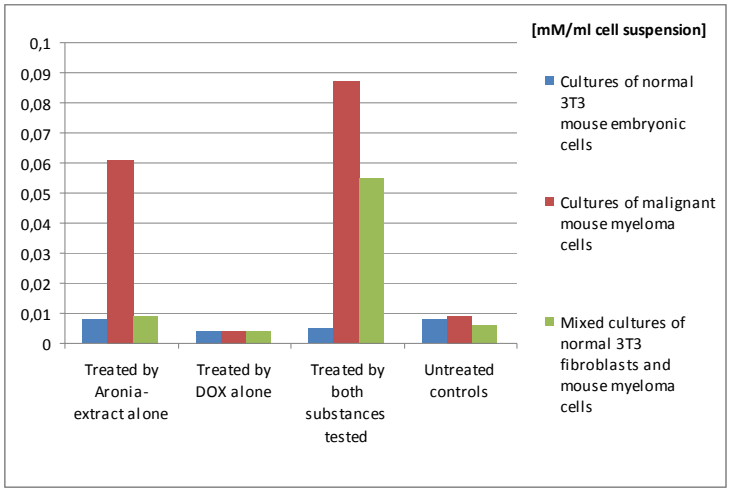

Figure 1: Influence of Doxorubicin and *Aronia melanocarpa* total extract on the intracellular GSH levels [mM/ml cell suspension], in normal 3T3 mouse embryonic fibroblasts, mouse myeloma cells and mixed cultures

Despite the lack of statistic significance, decrease in the levels of GSH in normal embryonic fibroblasts, malignant cells and mixed cultures could be noticed in all cases of cultivation in the presence of the chemotherapeutic drug alone **(Fig. 1)**. Those levels were partially restored in the presence of *Aronia*-extract in the three groups of cell cultures, both in the presence and absence of Doxorubicin. Similar effects have been observed in investigation of other plant extracts or their components [9, 15, 20-22]. On the other hand, analogical protective action by natural plant antioxidants has been established on the toxicity, induced by Doxorubicin [23], but also by other chemical drugs [24-26].

Statistically significant differences in GSH levels were observed in probes from the three different organs of experimental rodents from the four groups tested: treated by *Aronia*, DOX, (DOX+*Aronia*) and control group of Balb/c mice, respectively **(Fig. 2)**. DOX treatment caused significant reduction in GSH content, compared to controls in all investigated organs. *Aronia* pre-treatment, however, restored in part GSH level, but it did not reach those of the control group. There was not assessed a statistically significant difference in GSH content between *Aronia* group and control group.

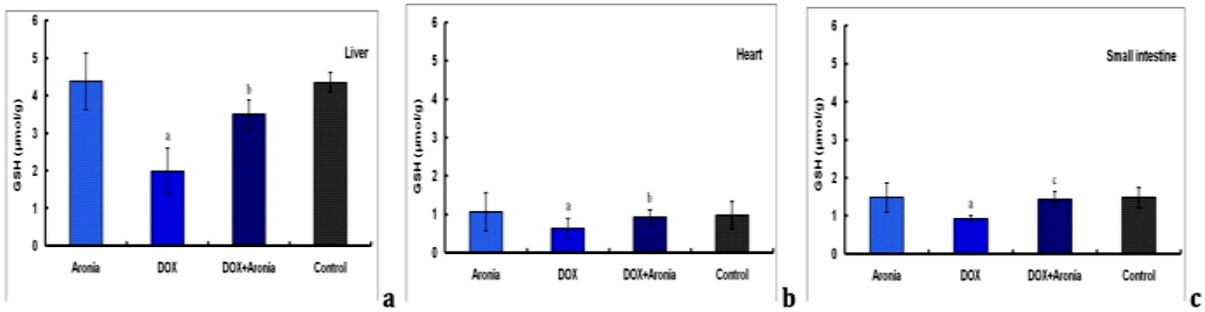

Figure 2: GSH levels [μM/g wet tissue] of DOX-treated mice with and without *Aronia melanocarpa* total extract pre-treatment, in liver (a); heart (b) and small intestine (c): Significantly different from control group ($p<0.001$): Significantly different from DOX-treated group ($p<0.001$); significantly different from DOX-treated group ($p<0.05$)

Values are expressed as mean±S.D., n=6

It is evident that 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 amounts are 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, has been characterized as the main site of GSH synthesis [27]. 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 major source for the intestinal mucosa, where its concentration is relatively high. According our results, 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. 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. The improvement of GSH-associated metabolism is a major mechanism for cellular protection against agents, which are known to generate oxidative stress. It is becoming increasingly apparent that GSH is central to a complex multifaceted detoxification system, where there is substantial inter-dependence between separate component members. Also, this tri-peptide participates in detoxification at several different levels, and it may scavenge free radicals, reduce peroxides or be conjugated with electrophilic compounds. Thus, it provides the cell with multiple defenses not only against ROS, but also against their toxic products.

CONCLUSIONS

Taking in consideration the results obtained in the current study, as well as respective literature data, further studies, directed to investigation on the immunomodulatory influence of *Aronia*-extract, but also the effects of its separate antioxidant components (polyphenols and anthocyanins) on the levels of intracellular GSH both *in vitro* and *in vivo*, should be provided. Also, further studies on GSH-dependent enzymes representing coordinately regulated defense mechanisms against oxidative stress, are necessary.

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