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Chemotherapy modulates the biological activity of breast cancer patients plasma: The protective properties of black chokeberry extract

Magdalena Kędzierska [a,b], Joanna Malinowska [a], Bogdan Kontek [a], Joanna Kołodziejczyk-Czepas [a], Urszula Czernek [b], Piotr Potemski [b], Janusz Piekarski [c], Arkadiusz Jeziorski [c], Beata Olas [a,*]

[a] Department of General Biochemistry, Faculty of Biology and Environmental Protection, University of Lodz, Pomorska 141/3, 90-236 Lodz, Poland
[b] Department of Chemotherapy, Medical University of Lodz, Copernicus Memorial Hospital, Lodz, Poland
[c] Department of Surgical Oncology, Medical University of Lodz, Copernicus Memorial Hospital, Lodz, Poland

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ABSTRACT

In breast cancer patients (before and during anti-cancer therapy) oxidative/nitrative damage to various molecules is observed. Furthermore, anti-cancer treatments may also influence the hemostatic properties of blood platelets and plasma. The aim of our study was to assess the effect of oxidative/nitrative stress (estimated by measurements of the levels of carbonyl groups and 3-nitrotyrosine in proteins – ELISA and C-ELISA methods, respectively; lipid peroxidation and total antioxidant level – TAS) on the selected parameters of hemostatic activity of plasma (the process of fibrin polymerization and lysis) collected from breast cancer patients after surgery and after various phases of chemotherapy (doxorubicin and cyclophosphamide). Subsequently, we also evaluated the level of oxidative/nitrative stress and hemostatic activity in plasma from these patients in the presence of the commercial extract of *Aronia melanocarpa* (Aronox®) *in vitro*. Patients were hospitalized in Department of Oncological Surgery and Department of Chemotherapy in Medical University of Lodz, Poland. We observed increased levels of biomarkers of oxidative/nitrative stress in plasma from patients with breast cancer (before or after surgery and after various phases of chemotherapy) in comparison to healthy group. Our further experiments demonstrated the hemostatic activity of plasma from the investigated patients differs from hemostatic properties of plasma obtained from healthy volunteers. We also recognize the existence of a relationship between oxidative stress (measured by the level of carbonyl groups) and changes of hemostasis in breast cancer patients after I and IV phases of chemotherapy. Moreover, the obtained results showed that the commercial extract from *A. melanocarpa* berries significantly reduced, in *in vitro* system, the oxidative/nitrative stress and hemostasis changes in plasma from breast cancer patients, after surgery and different phases of chemotherapy. Considering the data presented in this study, we suggest that the oxidative/nitrative stress in plasma obtained from breast cancer patients (not only before or after the surgery, but also after various phases of doxorubicin and cyclophosphamide chemotherapy) may induce changes of hemostatic activity, which may contribute to thrombosis in these patients. Our results also suggest that the commercial extract of *A. melanocarpa* may be regarded as a promising new source of bioactive antioxidant natural compounds for breast cancer patients.

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1. Introduction

The overproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS) is associated with both etiology and pathophysiology a variety of cancers. The dysregulation of ROS/RNS metabolism in cancer patients has been confirmed by results of investigations of several oxidative/nitrative stress markers, including products of lipid peroxidation, 3-nitrotyrosine and the enhanced formation of protein carbonyl groups in cancer cells, plasma and various blood cells (Gonenc et al., 2006; Delimaris et al., 2007; Erten-Sener et al., 2007; Kasapović et al., 2008; Hamo-Mahmood et al., 2009; Kędzierska et al., 2009, 2012a,b). Results of our earlier study also indicate that in breast cancer patients (before and after surgical interventions), the oxidative/nitrative stress exists (Kędzierska et al. 2012c, 2013). Moreover, during cancer therapy, various anti-cancer drugs or their combinations induce oxidative stress and affect some biological properties of crucial hemostatic elements, i.e., blood plasma and platelets (Olas and Wachowicz, 2002; Kędzierska et al., 2012a,b; Kędzierska et al. 2012c, 2013). Thrombotic risk associated with chemotherapy (including doxorubicin treatment) has been frequently reported

* Corresponding author. Tel./fax: +48 42 6354484.
E-mail address: olasb@biol.uni.lodz.pl (B. Olas).

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(Goodnough et al., 1984; Kim et al., 2011). The aim of our study was to assess the effects of surgery and chemotherapy (combination of doxorubicin and cyclophosphamide) in breast cancer patients on oxidative/nitrative stress (estimated by measurements of the levels of protein carbonyl groups and 3-nitrotyrosine, lipid peroxidation and total antioxidant status – TAS) in plasma. Since the influence of chemotherapy on coagulometric parameters of blood plasma has not been exactly defined, the role of oxidative/nitrative modifications are often suggested in its hematological toxicity. Therefore, in present work we also examined the effects of oxidative/nitrative stress on the selected parameters of hemostatic activity of plasma (the fibrin polymerization and lysis) from these patients. Additional aspects of this study were the assessment of oxidative/nitrative stress and the examination of hemostatic parameters of blood plasma isolated from breast cancer patients, in the presence of a commercial plant extract of *Aronia melanocarpa* (black chokeberry; Aronox®) *in vitro*. Aronox®, an antioxidant, was added to the whole blood. After 15 min incubation of blood with Aronox® the plasma was obtain and than the whole assay was repeated. These assays were compared with normal volunteers plasma. Aronox® is a rich source of valuable phytochemicals (i.e. a variety of flavonoids and phenolic acids) that are responsible for the multifunctional biological action of this extracts, including its antioxidative activity (Olas et al., 2008a,b; Ryszawa et al., 2006; Kędzierska et al., 2009), as well as anti-platelet (Olas et al., 2008b; 2010), anti-cancer (Yaneva et al., 2002; Malik et al., 2003; Lala et al., 2006), and anticoagulant properties (Bijak et al., 2011; Malinowska et al., 2012). According to the available literature, fresh berries of *A. melanocarpa* display the highest antioxidant capacity among berries (antioxidant capacity, measured as oxygen radical absorbance capacity (ORAC), of chokeberries is about 160 μmol of Trolox equivalents/g) (Zheng and Wang, 2003; Wu et al., 2004; Kulling and Rawel, 2008). The most important thing is that, there is any unwanted and toxic effects of black chokeberry fruits, juice and extracts *in vivo* and also *in vitro* (Kulling and Rawel, 2008).

2. Patients and methods

2.1. Materials

5,5′-Dithio-bis(2-nitro-benzoic acid) (DTNB), and 2,4-dinitrophenylhydrazine (DNPH) were purchased from Sigma. Sheep anti-3-nitrotyrosine polyclonal antibody were from Abcam (Cambridge, UK). The biotinylated anti-goat/mouse/rabbit antibody and streptavidin–biotinylated horseradish peroxidase were from DAKO (Glostrup, Denmark). All other reagents were of analytical grade and were provided by commercial suppliers.

Stock solutions of *A. melanocarpa* extract (commercial product – Aronox® by Agropharm Ltd., Poland; batch no. 020/2007k) was made in H_2O at the concentration of 5 mg/ml; kept frozen and was used for experiments. The total concentration of phenolics in the phenolic-rich powder used in this study was 309.6 mg/g of extract, including phenolic acids (isomers of chlorogenic acid) – 149.2 mg/g of extract, anthocyanins (anthocyanin glycosides: cyaniding 3-galactoside, cyaniding 3-glucoside, cyaniding 3-arabinoside, cyaniding 3-xyloside) – 110.7 mg/g and flavonoids (quercetin glycosides) – 49.7 mg/g of extract (Olas et al., 2008a; Kędzierska et al., 2009). The HPLC separation of the phenolic rich extract from berries of *A. melanocarpa* was described previously (Olas et al., 2008a; Kędzierska et al., 2009).

2.2. Classification of different study groups

Blood samples were taken from 55 healthy volunteers (females). Median age of healthy volunteers was 42 years (range, 25–56 years; mean, 45.7 year; standard deviation ± 4.6), and patients with breast cancer who were hospitalized in Department of Oncological Surgery, Medical University of Lodz, Poland.

There were 47 women with invasive breast cancer in the studied group. Median age of patients was 55 years (range, 43–72 years; mean, 55.1 year; standard deviation ± 6.7). In 20 patients the breast cancer was located in the left breast; in remaining 27 patients – in the right breast. Ductal invasive carcinoma was a dominant histological type of breast cancer. It was diagnosed in 44 out of 47 studied patients. Remaining three carcinomas were of lobular invasive histology. In subgroup of 44 ductal invasive breast cancers, nuclear grade was classified as G1 in 3 cases, G2 in 23 cases, and was classified as G3 in remaining 18 cases. Median diameter of primary tumor was 24.5 mm (range, 13–80 mm; mean, 26.5 mm; standard deviation, ±5.3). In 21 patients no metastases were found in axillary lymph nodes. In remaining 26 patients, axillary lymph nodes were cancer-positive. The number of surgically removed cancer-positive lymph nodes ranged up to 24. Cancers in 19 patients were classified as estrogen-receptor-positive (ER+), while 16 of cancer incidence were progesterone-receptor-positive (PR+). The overexpression of human epidermal growth factor receptor 2 (HER2) receptor was found in 13 out of 47 studied breast cancers. There were 17 triple-negative cancers in studied group.

Based on well established prognostic markers: age, tumor size, tumor grade, number of lymph node metastases, vascular permeation, estrogen receptor/progesterone receptor (ER/PR) status, and HER-2 status postoperative patients are classified into different risk groups to determine who will benefit from adjuvant chemotherapy. All patients were required to have normal bone marrow reserve renal and liver function and a left ventricular ejection fraction (LVEF). Patients with a clinical history of active angina, myocardial infarction or other cardiovascular disease were excluded. After 3 weeks from the surgery, 47 women with invasive breast cancer had I phase of chemotherapy (doxorubicin 60 mg/m^2 intravenous injection (iv) day 1 (d1) + cyclophosphamide 600 mg/m^2 (iv) (d1); the chemotherapy cycled every 21 days).

Blood samples were taken from healthy subjects or patients not taking any medications or addictive substances (including tobacco or alcohol) keeping a balanced diet (meat and vegetables), with similar socio-economic background, using no antioxidant supplementation. Blood from breast cancer patients and healthy volunteers was collected onto sodium citrate (at the final concentration of 5 mM) and immediately centrifuged (2000*g*, 15 min) to get plasma. In other experiments, whole blood (from breast cancer patients and healthy volunteers) was also incubated for 15 min at 37 °C with *A. melanocarpa* extract (50 μg/ml). After the incubation, plasma was isolated, and then different biomarkers of oxidative/nitrative stress and hemostasis were analyzed. The final concentration of Aronox® (50 μg/ml) used in our experiments corresponds to the reference of physiological range of this bioactive extract in human plasma.

All patients and volunteers expressed their written informed consent for participation in this study. The protocol was passed by the Committee for Research on Human Subjects of the Medical University of Lodz number RNN/252/07/KB and RNN/488/10/KB.

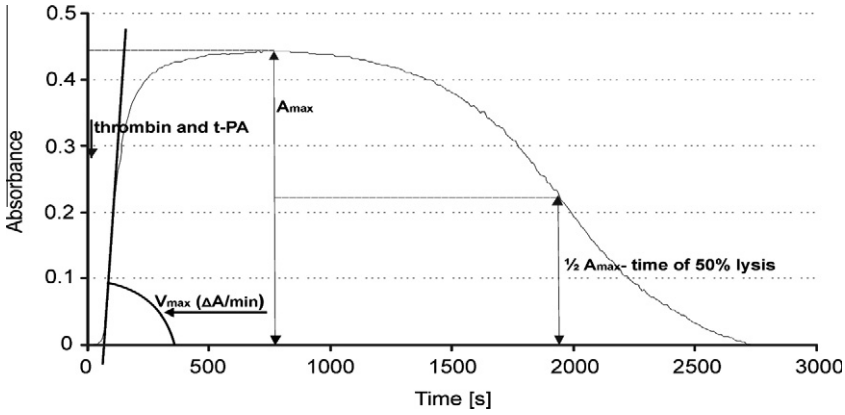

Fig. 1. The measurement of fibrin polymerization and lysis in whole human plasma (the maximal velocity, V_{max} [mOD/minute]; maximal absorbance (A_{max}) and the half-lysis time is defined as the time for the elastic modulus to decline to 50% its peak value (½ A_{max}).