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Indexes of the Redox State of Blood in Patients with Breast Cancer of Different Hormone Receptor Status

Anatolii Burlaka[1*], Anastasiia Vovk[1], Irina Ganusevich[1], Ihor Motuzyuk[2], Oleg Sidorchuk[2], Sergii Lukin[3], Sergii Virko[3]

[1]R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology NAS of Ukraine, Ukraine

[2]O.O. Bogomolets National Medical University, Kyiv, Ukraine

[3]V.E. Lashkaryov Institute of Semiconductor Physics, Kyiv, Ukraine

[*]**Corresponding author:** Anatolii Burlaka, Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology NAS of Ukraine, Ukraine. Tel: +380676834516; Email: apburlaka@gmail.com

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Abstract

In women of postmenopausal age, the level of circulating estrogen in blood serum and breast tissue is reduced, but the incidence of breast cancer in this female cohort remains high, indicating that there are other factors that contribute to the increase in morbidity.

Aim: To investigate the level of Ceruloplasmin [CP], Transferrin [TF], NO-Hemoglobin [NO-Hb], Methemoglobin [MetHb], superoxide and NO-generating activity of neutrophils and Matrix Metalloproteinases-2 And -9 [MMP-2 and – 9] in the blood of patients with breast cancer in relation with the hormone receptor status of tumors.

Object and Methods: We have studied the blood samples of patients of reproductive and postmenopausal age with breast cancer of stages II-III. The levels of CP, TF, "free" iron, hemichromes, MetHb, NO-Hb, ubisemiquinone, superoxide and NO-generating activity of neutrophils were investigated by the method of Electron Paramagnetic Resonance [EPR]. The level of active forms of gelatinases in blood serum was determined by zymography in a polyacrylamide gel.

Results: In patients of reproductive and postmenopausal age with BC of ER+/PR+ and ER-/PR- status, the level of CP exceeded the corresponding index in healthy donors, and the level of TF was significantly lower than in control group. An increase in NO-Hb content in blood was observed, this index was the highest in patients of post-menopausal age with ER-/PR- status and directly correlated with NO-generating activity of neutrophils [$r = 0.56$; $p < 0.05$]. The activity of MMP-9 in the serum of patients with ER-/PR- status was significantly higher [by 3.3 times] than in patients with ER+/PR+ status. In the blood of 78% of postmenopausal patients with breast cancer of ER-/PR- status, an increased content of estrogen metabolites - quinones and ubisemiquinones was found, the latter level was directly correlated with the stage of the tumor process ($r = 0.58$; $p < 0.05$). It has been established that neutrophils in the blood of patients of reproductive age with ER+/PR+ and ER-/PR- breast cancer generate SOR with a rate 9 and 18 times higher than in donors, respectively, and NO generation rate was more than 12 times higher in both subgroups.

Conclusion: We have detected the disturbed redox state of blood with the accumulation of compounds with hormonal and progenotoxic effects in patients with breast cancer, and such alterations depend on the hormone receptor status of tumors.

Keywords: Breast Cancer; Hormone Receptor Status; Nitric Oxide; Redox State Ofblood; Superoxide Radicals

Introduction

In women of postmenopausal age, the level of circulating estrogen in blood serum and breast tissue is reduced, but the incidence of breast cancer in this female cohort remains high, indicating that there are other factors contributing to the increased morbidity. In our opinion, such factors include the ability of estrogens and their metabolites to disturb equilibrium in the redox state in organs and blood by enhancing the generation of Superoxide Radicals (SOR) that exert both signaling and damaging effects. However, during menopause, the level of iron in the body increases due to its accumulation as a result of the cessation of blood loss and the degradation of iron-containing proteins [1-5]. The redox disturbance, the deposition and regulation of the level of iron, which under physiological conditions supports cell proliferation and growth, are the key factors in the survival of cancer cells in microenvironment of the tumor and metastases, and may contribute to the progression of breast cancer, which gives grounds for assessing their predictive value [5,6]. Matrix Metalloproteinases (MMPs) are known as enzymes that, in the process of tumor invasion and metastasis, provide proteolytic cleavage of the intercellular matrix and activation of a number of factors, chemokines and receptors by their proteolytic modulation [7]. Recently, the attention of many researchers is focused on the study of the relationship between the levels of expression/activity of MMPs and the hormone receptor status of tumors of patients with breast cancer. A number of modern studies assess gelatinase levels in relation to the ER/PR status of breast tumors in order to use these indexes for predicting the course of the disease [8,9]. This article provides an insight on the levels of redox-dependent molecules (ceruloplasmin, transferrin, hemichrome, Methemoglobin (MetHb), NO-Hemoglobin (NO-Hb), ubisemiquinone) and MMPs in patients with breast cancer, depending on the hormone receptor status of the tumors. The aim of the study was to investigate the level of Ceruloplasmin (CP), Transferrin (TF), NO-Hemoglobin (NO-Hb), methemoglobin (MetHb), superoxide- and NO-producing activity of neutrophils and matrix metalloproteinases-2 and -9 (MMP-2 and -9) in the blood of patients with breast cancer in relation with the hormone receptor status of tumors of patients with breast cancer of stages II-III.

Object and Methods of the Study

Blood samples of patients with breast cancer of stages II-III, of reproductive age (33.0 ± 6.0 years old, n = 40) or postmenopausal age (57.0 ± 5.0 years, n = 42) were studied. Receptor status of tumors in patients of reproductive age was ER+/PR+ in 23 cases, and ER^-/PR^- in 17 cases. In the postmenopausal age group, there were 27 and 15 cases, with ER+/PR+ and ER^-/PR^- status of breast cancer respectively. By the histological type, 100% of breast tumors were adenocarcinomas. The patients were treated at the National Cancer Institute of the Ministry of Health of Ukraine. The research was carried out in accordance with the principles of conducting biomedical research, set forth in the Helsinki Declaration of the World Medical Association. Patients were informed and provided their agreement on the use of clinical materials for research purposes. The levels of CP, TF, "free" iron, hemichromes, MetHb, NO-Hb, and ubisemiquinone were investigated by the method of Electron Paramagnetic Resonance (EPR) in a low-temperature mode (77 K) [10]. Samples were prepared using 0.5 ml of blood with the addition of Trilon B, and frozen in a special press form at 77 K. The rate of SOR generation by neutrophils was determined by the ESR method at room temperature in a paramagnetic-pure quartz dewar using the spin trap TEMPONE-H ("Sigma"). The NO-generating activity of blood neutrophils was determined by the EPR method using Spin Traps technology at a temperature of 77 K. Diethyldithiocarbamate ("Sigma") was used as a spin trap [11]. Neutrophils were isolated from blood according to the standard procedure [12]. The same indexes were assessed in blood serum of conditionally healthy women 35-63 years old (n = 17) and served as a control. EPR spectra were recorded at a temperature of liquid nitrogen (77 K) in a paramagnetic-pure quartz dewar using a computerized spectrometer PE-1307 with a resonator H_{011}. The power of the SHF source was 40 mW, the modulation frequency was 100 kHz and the amplitude 10 Gauss, the receiver's time constant $\tau = 0.3$ sec. The level of the studied indices is presented in Arbitrary Units (a.u.), compared with the intensity standard, represented by a specially oriented sample of ruby monocrystal (Al_2O_3) with low content of Cr^{3+} ions. The method of double integration was used to assess the concentration of molecules by comparison of the intensity of the signals in the EPR spectra with the intensity of the standard. Truncation error of spectrum integration and the deviation of spectrum reproduction of one sample did not exceed 3%. The level of active forms of gelatinases in blood serum was determined according to the method described in the work [13]. The data are presented as median values with standard error (M ± SE). The survival of the patients was analyzed by the Kaplan-Meier method, for the paired comparisons the log-rank criterion was used [14]. The statistical analysis was conducted using GraphPadPrism 6 and Excel software license applications. The difference between the scores was considered to be significant at $p < 0.05$.

Results and Discussion

Cerulloplasmin is a protein of the acute phase of inflammation and a multifunctional enzyme that exerts the activity of amino oxidase, superoxide dismutase, and ferroxidase. It catalyzes the oxidation of Fe^{2+} to Fe^{3+}, which is important for the loading of Apo-Transferrin (apo-TF) with iron ions. Ceruloplasmin is synthesized in the liver, but can also be produced by the breast cancer cells. The CP molecule consists of six domains and contains six ions of copper, three of which form a tri-nuclear cluster, and the other three are located separately in domains 2, 4 and 6 [1,15]. In

the human blood, the CP could be detected by the EPR method, and identified by the intensity of the EPR signal with g = 2.05, 2.209 (A = 155-200 Gs), which has a low-intensity component with g = 2.003 and a peak-peak width of 1.27 mT. EPR lines with g-factors in the range g = 2.00 and ΔHpp = 1.03-1.37 mT were observed in the EPR spectra of blood of approximately 78% of patients with breast cancer and were of a free radical nature. In patients of reproductive age with ER^+/PR^+ status of BC, it was found that the CP level was almost twice higher than the corresponding value in the group of donors (0.78 ± 0.08 a.u.) and reached 1.50 ± 0.13 a.u. while in patients with ER^-/PR^- status this idex was 1.06 ± 0.08 a.u. ($p < 0.05$) (Table 1).

Index	Donors	Patients with BC			
		Reproductive age		Postmenopausal age	
		ER^+/PR^+	ER^-/PR^-	ER^+/PR^+	ER^-/PR^-
Ceruloplasmin, a.u.	0.78±0.08	1.50±0.13	1.06±0.08	1.21±0.09	0.67±0.09
Transferrin, a.u.	0.81±0.10	0.34±0.08	0.47±0.07	0.71±0.10	0.98±0.15
NO-hemoglobin, a.u.	-	0.31±0.07	0.58±0.13	1.61±0.22	2.45±0.27
Superoxide-generating activity of neutrophils, nMole/10^5 cell·min	0.19±0.08	1.69±0.11	3.49±0.27	4.63±0.39	5.04±0.42
NO- generating activity of neutrophils, nMole/10^5 cells	0.23±0.03	2.75±0.24	2.93±0.30	3.26±0.29	4.17±0.35
MMP-2, a. u.	-	1.1±0.81	1.8±0.98	1.7±1.13	5.8±2.1
MMP-9, a. u.	-	1.4±0.93	4.7±1.2	1.4±0.97	4.4±1.1

Table 1: Indicators of redox-status of blood in patients with breast cancer.

In postmenopausal women with ER^+/PR^+ status of BC, the CP level exceeded the control values and was 1.21 ± 0.09 a.u. ($p < 0.05$), in patients with ER^-/PR^- status this index was 0.67 ± 0.09 a.u. and did not differ significantly from the values of the donors ($p > 0.05$). The normal level of CP is important for its function of copper transport in the tissue, oxidation of catecholamines and serotonin, antioxidant defence and anti-inflammatory action. In patients of reproductive and postmenopausal age with ER^+/PR^+ status of BC the level of CP is significantly higher than in patients with ER^-/PR^- status of BC, and an enhanced antioxidant defense in these patients can contribute to a positive response to chemotherapy. Transferin in the human body performs various functions, the main of which is the intracellular transport of iron ions required for the processes of cell growth, proliferation, differentiation, and apoptosis, and is a part of the system protecting the body from the accumulation of "free" iron. Apo-TF contains two iron ions, which bind to the protein molecule in the trivalent state (Fe^{3+}). At physiological conditions, only 30% of TF sites are loaded with iron ions [13,16,17]. In patients of the reproductive age with ER^+/PR^+ and ER^-/PR^- BC, TF levels were found to be 0.34 ± 0.08 and 0.47 ± 0.07 a.u., respectively, which was significantly lower than the control value [0.81 ± 0.10 a.u.] ($p < 0.05$) (Table 1). The level of TF in the blood of postmenopausal patients with ER^+/PR^+ and ER^-/PR^- BC status was 0.71 ± 0.10 and 0.98 ± 0.15 a.u., respectively, and was recorded with other spectral characteristics, acquiring the form of a singlet, which may indicate the binding of a larger number of iron ions to the protein molecule and a change of its conformation. Methemoglobin (MetHb, ferrideoxyhemoglobin in a high-spin state) is formed due to oxidation induced hemolysis and oxidation of Fe^{2+} in Hemoglobin (Hb) by superoxide radicals.

The level of MetHb in the blood of donors did not exceed 1-1.5% (0.08 ± 0.02 a.u.) of the total Hb level. In the blood of postmenopausal patients with ER^-/PR^- BC status its level increased up to 0.45 ± 0.07 a.u. ($p < 0.05$) due to the oxidation and accumulation of "free" iron during the cessation of menstruation (reduction of iron losses), which leads to overload of the body with this element [1,3]. This may also be caused by decomparentalization of iron ions due to the activation of oxidative damage to lipids, proteins, reductive Transformation of TF to apo-TF, and also the "exit" of deposited iron from ferritin [18,19]. But MetHb may undergo further degradation with the formation of various forms of hemichromes - low-spin MetHb forms, so its blood level in these patients may be altered in the direction of reduction with the accumulation of hemichromes. With the increase of the level of MetHb and "free" iron, hemic hypoxia occurs, the work of the Electron Transport Chain (ETC) of the mitochondria and endoplasmic reticulum is violated. Increased blood concentration of these molecules can be an important etiological factor in the development and progression of breast cancer, as they are the catalysts of autooxidation reactions and the subsequent increase of SOR generation. In 78% of postmenopausal patients with ER^-/PR^- BC status, there were detected EPR signals for MetHb (g = 6.0), free iron (g = 2.20-2.40), hemichromes (g=2.62) (g = 2.62), estrogen metabolites - quinones and ubisemiquinones (g = 2.00); the content of the latter increased and correlated directly with the stage of the tumor process ($r = 0.58$; $p < 0.05$). When the levels of Nitrogen Oxide (NO)

increase in the blood, it diffuses into erythrocytes and reacts with Hb [20,21]. There is an oxidation-reduction reaction in which NO forms a stable complex with Hb (NO-Hb), and bivalent iron is oxidized to trivalent. In patients of reproductive age with ER^-/PR^- status of BC, the level of NO-Hb (which has spectral characteristics g_1=2.07, g_2=1.98 and $g_{med.}$=2.01 and triplet splitting) was 0.58 ± 0, 13 a.u. and significantly exceeded that for ER^+/PR^+ group (0.31 ± 0.07 a.u.) ($p < 0.05$). In patients of postmenopausal age with ER^-/PR^- tumors, the level of this index was 2.45 ± 0.27 a.u., which was 1.5 times higher compared with patients with positive hormone receptor status (1.61 ± 0.22 a.u.) ($p < 0.05$) (Table 1). It should be noted that this index in blood donors was below detection limit.

Transport of NO with hemoglobin to a metabolically active tissue provides regulation of vasodilation and blood supply. This mechanism includes the covalent binding of NO to the cysteine residue in the β-chain of hemoglobin (Cysβ93) with the formation of Nitroso-Hemoglobin (NO-Hb) with subsequent NO transfer to the vascular endothelium, which may be a new therapeutic approach for treatment of the diseases associated with disturbed microcirculation perfusion and for assessment of the degree of oxygenation and deoxygenation of hemoglobin in various pathological conditions. NO-Hb can be in a high-spin (T), and a low-spin (R) states [2]. The highest NO-Hb level (up to 4.5 ± 0.87 a.u.) was determined in postmenopausal patients with BC of ER^-/PR^- status (n = 6), which was directly correlated with the level of NO-generating activity of neutrophils ($r = 0.56$; $p < 0.05$). The level of superoxide-generating activity of neutrophils in patients of the reproductive age with BC of ER^+/PR^+ and ER^-/PR^- status was 1.69 ± 0.11 and 3.49 ± 0.27 nMol/10^5 cells · min, which was 9 and 18 times higher than in donors (0.19 ± 0.08 nMol/10^5 cells · min). In postmenopausal patients with BC of ER^+/PR^+ status, this index was 4.63 ± 0.39 nMol/10^5 cells · min, and in ER^-/PR^- - 5.04 ± 0.42 nMol/10^5 cells · min., which was higher compared to patients of reproductive age and healthy donors ($p < 0.05$). The NO-generating activity of blood neutrophils of donors was 0.23 ± 0.03 nMol/10^5 cells see (Table 1), in patients of the reproductive age with BC of ER^+/PR^+ and ER^-/PR^- status - 2.75 ± 0.24 and 2.93 ± 0.30 nMol/10^5 cells ($p < 0.05$). In postmenopausal patients with ER^+/PR^+ and ER^-/PR^- status this index was 3.26 ± 0.29 and 4.17 ± 0.35 nMol/10^5 cells respectively. In the blood of patients with BC, the increase of nitric oxide levels is caused by its enhanced synthesis by iNOS in neutrophils, and under excessive levels of generation, it loses its protective functions and exerts vaso-depressant and cytotoxic effects. We determined the activity of gelatinases in the blood serum of patients of reproductive age and revealed that in patients with BC of ER^-/PR^- status, the median activity of MMP-2 was 1.8 a.u. and by 1.6 times higher than in patients with BC of ER^+/PR^+ status of breast cancer, but the difference between the groups was insignificant. The level of activity of MMP-9 in blood serum of patients with ER^-/PR^- status reached 4.7 a.u. that is 3.3 times higher than that in patients with ER^+/PR^+ status ($p < 0.05$) (Table 1). The level of MMP-2 and MMP-9 activities in the blood serum of postmenopausal patients significantly differed between ER^-/PR^- and ER^+/PR^+ groups.

Thus, the obtained data correlate with a number of other studies [22-26] and indicate the association of high levels of MMP-9 with hormone receptor status in breast cancer patients. Such a relation may result from a hormone-dependent control of the activity of gelatinases, which, given the above data on SOR generation, can be realized by redox activation of these enzymes [27,28]. The 5-year Overall Survival (OS) of patients with BC was analyzed, depending on the level of above mentioned indices. The analysis of OS depending on the level of NO-Hb in the blood revealed the statistically significant difference indicating the better survival rates in patients with a lower level of this index, regardless of age (Figure 1). In patients of reproductive Age (A) with NO-Hb levels above the median (> 0.38 a.u.), the 5-year OS rate was 43%, median survival was 39 months ($\chi 2 = 5.6$, $p = 0.02$). In patients of postmenopausal age (B) with a level of NO-Hb above the median (> 1.94 a.u.), the 5-year OS was 34%, median survival was 27 months ($\chi 2 = 3.6$, $p = 0.05$).

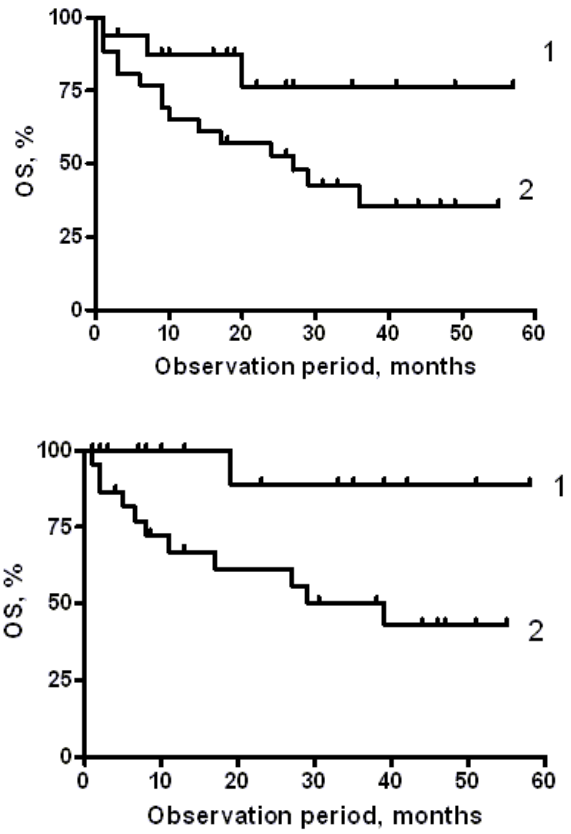

Figure 1: OS of patients of reproductive (A) and postmenopausal (B) age with breast cancer of stages I-III depending on the level of NO-Hb in the blood.

A. 1: < 0.38 a.u. (n = 18), the MS was not reached; 2: > 0.38 a.u. (n = 22), MS was 39 months; $\chi 2 = 5.6$, $p = 0.02$.

B. 1: < 1.94 a.u. (n = 17), the MS was not reached; 2: > 1.94 a.u. (n = 25), MS was 27 months; $\chi 2 = 3.6$, $p = 0.05$.

When combined with high levels of MetHb, hemichromes with locally high concentrations of estrogen in the mammary glands, activation of SOR generation and activation of MMPs can catalyze cyclic oxidation with degradation of microenvironment of cells. The metabolites of estrogen, quinones and ubisemiquinones, the levels of which increase and which are a source of SOR generation, can contribute to the disturbed redox state of the blood. Estrogens initiate SOR generation by influencing the activity of mitochondria, since the phenolic hydroxyl group in the C_3 position of the hormone may be oxidized by electron acceptance or may lose proton and act as a prooxidant. In addition, estrogens can be metabolized to catechols, which oxidize to quinones and ubisemiquinones in a redox cycle, generate SOR, causing mitogenic effects. Natural and synthetic estrogens cause a response reactions in concentrations with a clearly defined dose range. At elevated concentrations of estrogens, metabolic reactions that are harmless at their low levels can cause effects that include the oxidation of estrogens to the corresponding quinones and increased generation of SOR in the oxidizing cycle of the ethers, diethylstilbestrol and their quinones (Figure 2). The activity of enzymes that support cyclic oxidation of estrogens and the generation of SOR correlates with the initiation of tumor development in mammary glands [1,2,4]. Disturbed binding to transferrin, ferritin Fe^{3+}, and disturbed Fe^{2+} oxidation to Fe^{3+} by ceruloplasmin leads to the accumulation of "free" iron and hemichromes. In addition, estrogens, their metabolites and "free" iron activate the oxidation-associated signaling that promotes cell proliferation. CP can act as secondary messenger in cascades of intracellular signaling that induce and support the oncogenic phenotype of tumor cells.

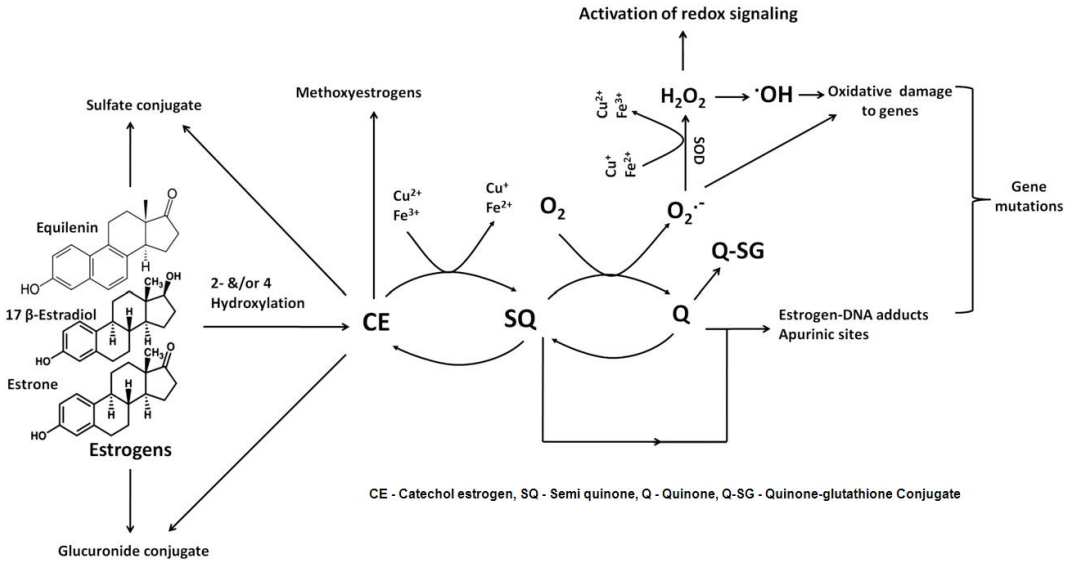

Figure 2: Scheme of the redox cycle of the metabolism of estrogens with the formation of oxygen radicals [29].

The signaling functions of CP and its metabolites are realized through the mitogen-Activated Protein kinase, AP-1 and NF-κB pathways responsible for gene transcription responsible for cell growth and transformation. Consequently, the accumulation of SOR, NO, NOHb, MetHb, hemichromes, ubisemiquinones with hormonal and progenotoxic effects, or the use of hormone replacement therapy that initiates a redox cycle, make postmenopausal women susceptible to developing breast cancer and its progression [1-7].

Conclusion

We have detected the disturbed redox state of blood with the accumulation of compounds with hormonal and progenotoxic effects in patients with breast cancer, and such alterations depend on the hormone receptor status of tumors. The redox-state of the blood characteristic for patients with breast cancer is formed by the increased superoxide and NO-generating activity of neutrophils, the appearance of significant levels of NO-Hb, MetHb, hemichromes, ubisemiquinones and gelatinase activity. The influence of these indices on the course of the disease and survival of patients with breast cancer was revealed.

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