

Breast Cancer. Screening Criteria

D. Zob¹, M. Vasilescu², M.I. Gruia², R. Anghel¹

¹Department of Medical Oncology I, Institute of Oncology, Bucharest, Romania

²Research Department, Institute of Oncology, Bucharest, Romania

Rezumat

Cancerul mamar. Criterii de monitorizare

Cancerul mamar este neoplazia cu frecvența cea mai mare la sexul feminin reprezentând cca 26% din toate neoplaziile diagnosticate anual, fiind a doua cauză de deces după cancerul pulmonar, la femei. Deși prognosticul cancerului mamar s-a ameliorat în ultimii ani, un număr important de paciente vor prezenta evoluție de boală și vor deceda din cauza acestei malignități - rezistentă la diverse citostatice reprezentând principal cauză de eșec terapeutic la peste 90% din pacienții cu această localizare. Întrucât evaluarea genică a rezistenței la chimioterapie este greu de realizat în condițiile practicii clinice curente, am dorit urmărirea unor parametri ușor de evaluat, care s-ar putea corela cu rezistența la chimioterapie. În cele ce urmează, vom prezenta un caz clinic al unei paciente cu cancer mamar cu rezistență la chimioterapie cu factori de prognostic nefavorabili, căruia i-am determinat parametrii biochimici de stres oxidativ (malondialdehida serică - marker al peroxidazei lipidice, ceruloplasmina serică - a cărei activare determină supraproducție de specii reactive de oxigen, grupările thiol albuminice plasmatice - componente semnificative ale apărării antioxidante, antioxidanți totali plasmatici - ce măsoară abilitatea plasmiei de a rezista efectelor oxidative ale speciilor reactive de oxigen) corelați cu evaluarea apoptozei (moarte celulară programată) pe țesut tumoral proaspăt. S-au corelat datele histopatologice, imunohisto-

chimice și determinările flowcitometrice. Chiar dacă rezultatele obținute indică instalarea unui stres oxidativ și diminuarea apărării antioxidante endogene, se pare că acest stres oxidativ nu a fost suficient pentru inducerea citotoxicității tumorale dorite.

Cuvinte cheie: cancer mamar, rezistența la chimioterapie, evaluare

Abstract

Breast cancer is the most frequent type of neoplastic disease in women, amounting to approximately 26% of all annually diagnosed cancers, and representing the second leading cause of female death after pulmonary cancer. Although the prognosis of the disease has improved in recent years, an important number of patients will suffer an evolution of the disease and will die due to this malignancy, which remains resistant to various cytostatic drugs, this being the main cause for therapeutic failure in over 90% of patients with a neoplastic development at this level. As a genic evaluation of the resistance to chemotherapy is difficult to obtain under the current clinical practice conditions, we desired to follow a series of parameters which were easy to evaluate, and which could be correlated with resistance to chemotherapy. Further on, we shall report a clinical case of a patient presenting with chemotherapy resistant breast cancer and unfavourable prognostic factors, in which we determined the biochemical parameters for oxidative stress (serum monoaldehyde - a marker of lipid peroxidase, serum ceruloplasmin - whose activation stimulates an overproduction of reactive species of oxygen, plasmatic albumin thiol groups -

Corresponding author:

Daniela Zob, MD
Department of Medical Oncology I,
Institute of Oncology, Bucharest, Romania
E-mail: danielazob@yahoo.com

important components of antioxidant defence, total serum antioxidants – which measure the ability of the plasma to resist in face of the effects of reactive species of oxygen), correlated with an apoptosis evaluation (scheduled cellular death) performed on fresh tumoral tissue. Histopathological, immunohistochemical and flowcytometry data correlated. Even if the results obtained indicate a high oxidative stress status and a diminished capacity of endogenous antioxidant defence, it appears that this oxidative stress alone was not potent enough to induce the desired tumoral cytotoxicity.

Key words: breast cancer, resistance to chemotherapy, evaluation

Introduction

The medical literature is quite rich in theories according to which free radicals and other reactive oxygen species (ROS) are involved in human pathology. (1) The contribution of these ROS in the pathology of various diseases may be significant or not, which is why it is highly important to establish the degree to which these compounds participate in the production of lesions which are typical for different types of afflictions. (2) In principle, the diseases associated to oxidative stress could result through one, or both, of the following ways:

- a) a decrease in antioxidant defence by genetic mutations which affect antioxidant enzymes (such as CuZnSOD, MnSOD, glutathione-peroxidase-GPX) or due to a depletion of antioxidants during the evolution of another disease. (3)

Many xenobiotics are metabolised by means of conjugation with glutathione (GSH). The excess of xenobiotics entering the organism determines a depletion of GSH, generating oxidative stress, even if the respective xenobiotic is not directly producing ROS. (4)

The depletion of antioxidants or of other essential nutritious elements, due to an inadequate diet, can also lead to such oxidative stress. (5,6)

- b) an increase in ROS production, either through exposure to high concentrations of O₂, or due to the presence of various toxins that can even be reactive species themselves (ex. NO₂[•]) - or can be metabolised to ROS, or through excessive activation of the natural systems which produce ROS (for example, an excessive activation of phagocytic cells in chronic inflammatory diseases). (7)

The second mechanism is much more relevant in the study of diseases and represents the target of therapeutic interventions, while the first type of mechanism is usually neglected, very little importance being allotted to the nutritional status of patients.

A malignant cell acts in according to a dual 'cause and effect' type of relationship, without it being possible to state of a surety whether the oxidative stress at this level is a cause

or a consequence of the malignant transformations taking place.

Although the intense oxidative stress generated during radiotherapy might be efficient in inducing tumoral cytotoxicity, the moderate oxidative stress generated during chemotherapy might have opposite effects, contributing to the development of therapy resistance mechanisms in tumour cells, with a consequent decrease in treatment efficacy. (8)

Further on, we shall present a clinical case of a patient presenting with chemotherapy resistant breast cancer and unfavourable prognostic factors, in which we determined the biochemical parameters for oxidative stress, correlated with an apoptosis evaluation performed on fresh tumoral tissue.

As a genic evaluation of the resistance to chemotherapy is difficult to obtain under the current clinical practice conditions, we desired to follow a series of parameters which were easy to evaluate, and which could be correlated with resistance to chemotherapy.

Case report

Patient NM, 38 years of age, was diagnosed through tumour biopsy puncture on the 21st of September 2010 with stage III left side breast cancer (carcinomatous mastitis). HPB 1220807-808/ IOB/21.09.2010 - invasive ductal carcinoma. The thorax, abdomen and pelvis CT scans revealed the left mammary tumour; large left axillary adenopathies, the largest being 4-5 cm in diameter, with no secondary lung determinations, no pleural or pericardial effusion, diffuse liver steatosis and liver enlargement, with no space replacing processes and no adenopathies at the superior abdominal level. Normal aspect of the spleen, pancreas and kidneys. These evaluations were performed at the Bucharest Emergency Hospital.

Between 18.10.2010 - 02.03.2011, she was submitted to seven series of neoadjuvant polychemotherapy with Epirubicin, Cyclophosphamide and 5 Fluorouracil, followed by other four series of Gemcitabine, Paclitaxel and Epirubicin, and, due to the inconclusive response to therapy, with a persistence of the inflammatory phenomena, neoadjuvant external irradiation by Cobalt-therapy at the level of the breast and of the lymphatic areas was opted for, in a therapeutic dose of 50 Gy, to which the patient was submitted between 23.03.2011- 26.04.2011.

As the inflammatory phenomena were remitted, on the 9th of June 2011, a surgical intervention was performed, practicing radical left mastectomy, with axillary lymphodissection of the first, second and third group of axillary nodes. Histopathology biopsy revealed invasive ductal carcinoma, G3, 15 nodes examined, out of which 10 nodes were invaded by tumoral emboli in the lymphatic vessels ypT3, ypN3a. (9)

Specific immunohistochemical tests showed negative ER in the tumoral cells, as well as negative PR, positive discontinuous membrane CerbB2 moderately active in <10% of the tumour cells, 80-85% positive Ki 67.

Postoperatively, it was decided that chemotherapy was to be resumed, and other 3 series of Gemcitabine, Paclitaxel

and Epirubicin were administered between 25.07.2011-03.10.2011.

On the 25th of October 2011, the patient was readmitted under suspicion of local recurrence and permeation nodules surrounding the scar, and an excisional biopsy under local anaesthesia was performed. HPB revealed carcinomatous infiltration at dermic level. On the 16th of the same month the patient was submitted to an evaluation CT scan in order to determine the current extent of the disease, which confirmed local recurrence surrounding the subaxillary scar and on the anterior parasagittal wall, tumoral right axillary and left subclavicular adenopathies, with no pulmonary or hepatic secondary determinations.

On the 21st of November 2011 chemotherapy was resumed, with Capecitabin and Navelbine. Under treatment, the patient presented clinical progression at the level of the permeation nodules, and irradiation of the thoracic wall is decided for. Electron radiotherapy is performed at the level of the permeation nodules surrounding the scar between 02.05.2012 - 15.05.2012 (30Gy therapeutic dose).

Since the patient presented progression of the disease under various different lines of chemotherapy, it is considered that the breast cancer is resistant to this type of treatment, and after the request of an informed consent, a tumour fragment is extracted for analysis (a permeation nodule) in May 2012, as well as blood samples in order to determine the biochemical parameters for oxidative stress.

Pulmonary metastases develop – visible on the pulmonary X-ray and 2 other series of Paclitaxel and Cisplatin are administered between 22.05.2012 - 21.06.2012, but the evolution is unfavourable, the patient developing pleurisy (thoracic CT 20/07/2012 - Fig. 1, 2, 3) and a second blood sample, to be analysed for oxidative stress, is drawn. Later, the patient presented respiratory failure followed by death, 2 months after the last dose of chemotherapy administered.

Determining the biochemical parameters of oxidative stress

1. Malondialdehyde values (MDA) – a marker of lipid peroxidation

MDA – the final product of lipid peroxidation was determined through the Carboneaumethod (10) Although controversial, this method has been largely utilised. The most difficult problem consists in identifying the carbonyl products that react with TBA, out of which MDA represents only a share. Studying the specific extinctions at 532 nm of the carbonylic compounds resulting from oxidative degrading of lipid hydroperoxides, it can be considered, without error, that the absorption at 532 nm is due to the TBA – MDA complex.

Normal values: 0 – 2 $\mu\text{mol}/100\text{ ml}$ serum (Fig. 4).

The recorded values indicate an increase in free radical production, which initiates the lipid peroxidation reaction. The increase is significant and is associated with the induction of a powerful, irreversible oxidative stress.



Figure 1. Thoracic CT scan 20.07.2012, pleural effusion, secondary pulmonary mass



Figure 2. Thoracic CT scan 20.07.2012, pleural effusion



Figure 3. Thoracic CT scan 20.07.2013, pleural effusion, secondary pleural mass

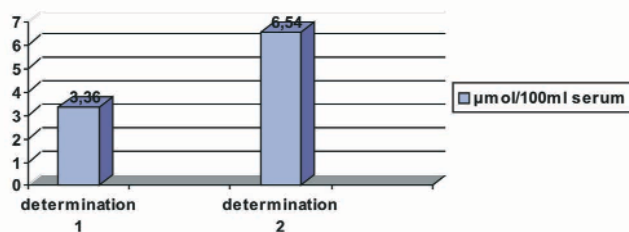


Figure 4. Malondialdehyde values (MDA)

2. Ceruloplasmin determination

The liver is the main site of ceruloplasmin synthesis, but it can be synthesized extrahepatically as well. The catalytic oxidation of Fe^{2+} or of Fe^{2+} containing compounds is called ferroxidase activity. Several methods for determining the ferroxidase activity of ceruloplasmin are described. We used the Ravin method, by means of the reaction of *p*-phenylenediamine in an acetic acid – acetate tampon. (11)

The values are expressed in I.U. The quantity of enzyme required to convert 1 μmol of substrate per minute is defined as 1 unit of ceruloplasmin. Normal values: 80- 120 I.U. ceruloplasmin (Fig. 5).

An increase in the Copper-oxidative activity of ceruloplasmin is registered, suggesting thusly an overproduction of reactive species of oxygen, which might be due to the metabolising of cytostatic drugs.

3. Determining albumin thiol groups

Determination of albumin thiols was performed by the Albini method, with DTNB.

Thiol groups are important components of the serum antioxidant defence system, being able to delay or cancel the progression of oxidative processes by inactivating alkoxy (RO^*) and hydroxyl radicals (OH^*) and transforming them into alcohol molecules, and water respectively, which are harmless.

In addition, the oxidized thiol radicals react among themselves, forming disulphide bonds.

Thus, through self-oxidation, the thiol groups fight the attack of several far more toxic free radicals, which can severely harm the tissues. The newly formed disulphide bonds can determine an instability in protein molecules and alter their functions.

Serum SH group determination tests are based on the ability of the SH group to form a coloured complex, which can be measured with a spectrophotometer, with a maximum absorbance rate at 412 nm, as a result of reacting with 5,5-dithiobis-2-nitrobenzoic acid (DTNB) at room temperature. (12)

Normal values 370 – 450 $\mu\text{mol/l}$. A lower value is directly correlated with a decrease in the efficiency of the serum antioxidant barrier formed by the thiol groups. (Fig. 6)

The results obtained show a decrease of thiol groups, suggesting a diminishing of the endogenous antioxidant fighting ability.

4. Determining total antioxidants

There are a series of tests that measure the antioxidant effect of non-enzyme defence in biological fluids, that can be used to determine the resistance index to oxidative attack. The majority of tests measuring the total antioxidant power used nowadays measure the ability of the plasma to resist the oxidative effects of reactive species generated in reaction mixtures. The technique that we used is based in terms of method on using reductants in redox related colorimetric

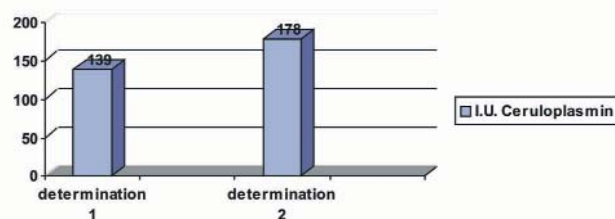


Figure 5. Determining the copper-dependent oxidase activity of ceruloplasmin.

(I.U. of ceruloplasmin copper-dependent activity)

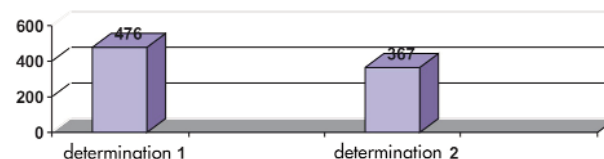


Figure 6. Determining albumin thiol groups

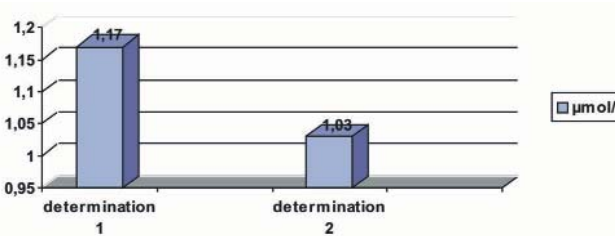


Figure 7. Determining total antioxidants

tests, employing an oxidant easily reducible in stoichiometric excess. (13,14)

Normal values are included in the interval 0.9-1.4 $\mu\text{mol/l}$. (Fig. 7)

A decrease in total antioxidant level is observed, suggesting the progression to an oxidative stress tilting the scale towards excess production of reactive species of oxygen, to the detriment of endogenous antioxidant fighting ability.

As we have already mentioned in the beginning, it is difficult to establish whether this oxidative stress is a cause or a consequence of the presence of malignant cells and of tumoral tissue, but it can be stated that once progressed, it has a devastating effect not only on the tumoral cells, where its cytotoxic effect is of benefit, but also on the other surrounding normal cells. Reactive species of oxygen, especially radicals, are highly reactive species, and can interact with any biochemical cellular component. However, due to their importance in the cellular metabolism, their interactions with nucleic acids, proteins, lipids, are those that produce alterations of vital cellular functions. The primary effect induced is local and reversible, depending on the degree of oxidative stress and on the level of antioxidants. This primary effect may last for years, and it can be compensated or prolonged. The transition to the secondary effect marks the irreversible stage. If in the primary stage only minor symptoms can be observed, development of secondary

effects is accompanied by clinical manifestations which are due to cellular lysis, altered functions, low resistance to effort, or infections. An associate disease status becomes obvious. (15,16)

In the screening of toxicity induced by chemotherapeutic drugs, ionising radiations or mutagenic chemical agents, apoptosis – scheduled cellular death, is a main marker of sensibility, representing also the mechanism for tumour cell destruction which has been best characterized.

Apoptosis induction results in the activation of endonucleases that cleave nuclear DNA. DNA fragmenting is known as a biochemical marker for apoptosis, allowing the selection and quantification of apoptotic cells by measuring the DNA quantity by means of flowcytometry. As a result of this fragmenting, the cells suffering apoptosis lose a part of their DNA due to detergent permeabilization, or to alcohol or acetone fixing. By chlorination with IP (fluorochrome that passes through the plasmatic membrane, is stoichiometrically intercalated among the base pairs of double stranded DNA and emits red fluorescence under laser beam stimulation) apoptotic cells are detected based on their lower quantity of hypodiploid (subdiploid) DNA, corresponding to a sub-G1 peak on the DNA quantity distribution diagram. (17)

The samples were processed in order to obtain cellular suspension, and the nuclei were marked with propidium iodide in a hypotonic tampon to evaluate the apoptotic status. Sample readings were performed with a FACSCalibur flowcytometer (the CellQuest program), and data analysis to determine the percentage of apoptotic cells was conducted by means of the WinMDI program. 10.000 events were acquired for analysis. The histogram charts obtained represent the DNA quantity distribution and are expressed depending on the fluorescence determined by the FL2-H detector and by the number of cells. (Fig. 8)

Apoptosis specific cellular DNA fragmenting allows highlighting of a subdiploid DNA peak by means of flowcytometry, corresponding to the percentage of cells bearing apoptotic status (18.78%).

The use of flowcytometry has allowed us to characterize the biopsy tumour fragment in terms of DNA quantity. This technique allows the detection of cellular clones presenting even small differences in terms of DNA quantity and offers data referring to the ploidy of the DNA; the ploidy level is expressed by the DNA index (DI), which is well correlated with the number of chromosomes determined through karyotype analysis. On the DNA quantity distribution histogram the existence of two populations of DNA can be observed: a major one presenting diploid DNA and a minor population with subdiploid DNA.

The tumour cell population specific proliferative activity, expressed by means of the S-phase cell fraction and the proliferation index, is high. (Fig. 9)

The measuring of DNA quantity of tumour cells and of the percentage of cells that have entered the phases of the cellular cycle through flowcytometry indicated, along with the diploid population, the presence of an aneuploid population ($DI=1.44$) with a high proliferation index (phase S=30.90%, G2/M = 20.58%).

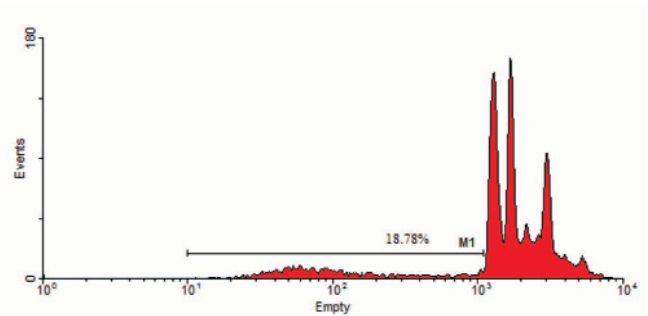


Figure 8. Apoptosis specific cellular DNA fragmenting allows highlighting of a subdiploid DNA peak by means of flowcytometry, corresponding to the percentage of cells bearing apoptotic status (18.78%)

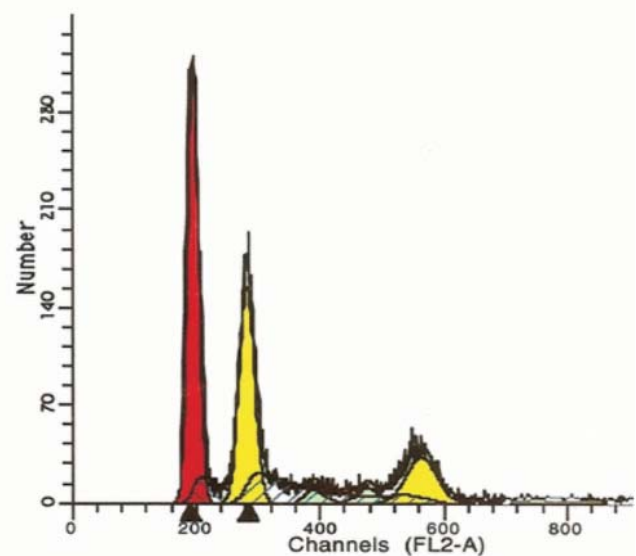


Figure 9. The measuring of DNA quantity of tumour cells and of the percentage of cells that have entered the phases of the cellular cycle through flowcytometry indicated, along with the diploid population, the presence of an aneuploid population ($DI=1.44$) with a high proliferation index (phase S=30.90%, G2/M = 20.58%)

Discussions

Correlation of histopathological data with those resulting from the immunohistochemical analysis and flowcytometry determinations suggests the presence of an aggressive tumoral tissues, with a high rate of proliferation. By administering cytostatic drugs, we waited for the cellular and molecular mechanisms of cytotoxicity to be mediated by the excessive production of reactive species of oxygen, and for these to significantly modify lipids and proteins through their destructive attack, thus destroying vital essential macromolecules and leading to the desired cytostatic and oncostatic effect.

Even though the results obtained indicate the development of oxidative stress, through increased production of free

radicals (measured by lipid oxidative degradation and Copper-oxidative activity of ceruloplasmin) and diminishing of endogenous antioxidant defence (measured by determining serum ability to reduce iron and by total thiol groups), it appears that this oxidative stress was not potent enough to induce the desired cytotoxicity alone. In this context, there are various assumptions regarding the biochemical mechanisms that lead to the resistance to treatment, and these are: (1) the tumour tissue does not metabolise enough oxygen, it has anaerobic tissue properties, and therefore free radical production at this level does not induce the desired toxicity. (2) the decrease in total thiol groups produced mainly through albumin oxidative degradation proves a presumed decrease in circulating albumin quantity, meaning in cytostatic compound transporters towards the periphery. Their decrease can represent a mechanism of transport inefficacy and of their effect. (3) There is a possibility that treatment inefficacy is due to the expression of various genes involved in resistance to treatment mechanisms. Their presence is preferable to be determined in the primary tumour, for treatment conduct orienting. As these procedures involve high costs and cannot be used routinely, we are looking to identify new markers to be used in determining resistance to treatment.

References

1. Shargorodsky M, Debbi O, Matas Z, Zimlichman R. Effect of long-term treatment with antioxidants (vitamin C, vitamin E, coenzyme Q10 and selenium) on arterial compliance, humoral factors and inflammatory markers in patients with multiple cardiovascular risk factors. *Nutr Metab (Lond)*. 2010;7:55.
2. Basaga HS. Biochemical aspects of free radicals. *Biochem Cell Biol*. 1990;68(7-8):989-98.
3. Gutteridge JM. Biological origin of free radicals mechanisms of antioxidants protection. *Chem Biol Interact*. 1994;91(2-3):133-40.
4. Kaplowitz N, Fernández-Checa JC, Kannan R, Garcia-Ruiz C, Ookhtens M, Yi JR. GSH transporters: molecular characterization and role in GSH homeostasis. *Biol Chem Hoppe Seyler*. 1996;377(5):267-73.
5. Appel LJ, Miller ER 3rd, Jee SH, Stolzenberg-Solomon R, Lin PH, Erlinger T, et al. Effect of dietary patterns on serum homocysteine: results of a randomized, controlled feeding study. *Circulation*. 2000;102(8):852-7.
6. Birben E, Sahiner UM, Sackesen C, Erzurum S, Kalayci O. Oxidative stress and antioxidant defence. *World Allergy Organ J*. 2012;5(1):9-19. Epub 2012 Jan 13.
7. Halliwell B, Gutteridge JMC. Free radical in biology and medicine. Third edition. Oxford university press. 1998.
8. Yu R, Schellhorn HE. Recent applications of engineered animal antioxidant deficiency models in human nutrition and chronic disease. *J Nutr*. 2013;143(1):1-11. Epub 2012 Nov 21.
9. Vasilescu C. Current problems in surgical oncology: 1. Theoretical models in oncology and their implications in surgical therapy. *Chirurgia (Bucur)*. 2011;106(1):17-22. Romanian
10. Carboneau MA. Peroxides measurement in blood and plasma. *Clinic. Chem*. 1991;37:1422-8.
11. Sunderman FW Jr, Nomoto S. Measurement of human serum ceruloplasmin by its p-phenylenediamine oxidase activity. *Clin Chem*. 1970;16(11):903-10.
12. Albini A. Standardization of protein free SH groups in blood plasma. *Boll. Soc. Ital. Sperim*. 1990;18:1829-98.
13. Olinescu R, Matachescu C, Nita S. The study of the oxidative potential of iron by coupled chemiluminescence. *Rom J Biophys*. 1993;2(3):95-106.
14. Olinescu R, Matachescu C, Nita S. A simple chemiluminescent system for the determination of total antioxidative capacity. *Rom. J Biophys*. 1992;2(4):245-53.
15. Reinisch N, Kiechl S, Mayr C, Schratzberger P, Dunzendorfer S, Kähler CM, et al. Association of high plasma antioxidant capacity with new lesion formation in carotid atherosclerosis; a prospective study. *Eur J Clin Invest*. 1998;28(10):787-92.
16. Barrera G. Oxidative stress and lipid peroxidation products in cancer progression and therapy. *ISRN Oncol*. 2012;2012:137289. Epub 2012 Oct 17.
17. Pozarowski P, Darzynkiewicz Z. Analysis of cell cycle by flow cytometry. *Methods Mol Biol*. 2004;281:301-11.