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ASSESSMENT AND CORRELATION OF OXIDATIVE STRESS BETWEEN HEALTHY ADULTS AND ADULTS PATIENTS WITH CHRONIC MYELOID LEUKEMIA TREATED WITH IMATINIB

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Abstract

Reactive oxygen species (ROS) are oxygen-containing radicals essential for cell signaling and other vital physiological functions. However, their increased production to an excessive amount can cause alterations in the cellular redox status with consecutive disruption of various normal biological functions.

Oxidative stress (OS) occurs when there is an imbalance between ROS production and antioxidant defence mechanisms.

Chronic OS results in many DNA modifications, and alterations in DNA repair, leading to DNA lesions of which many can be toxic and/or mutagenic. It is proven that OS is involved in the pathogenesis of chronic myeloid leukaemia (CML), a myeloproliferative neoplasm characterized by uncontrolled proliferation of maturing and mature myeloid cells, caused by the presence of translocation t(9;22) leading to the abnormal BCR-ABL fusion protein.

Historically treatment options for this disease were limited, with allogeneic stem cell transplant being the only potential curative therapy, but still with poor prognosis. Fortunately, the introduction of tyrosine kinase inhibitors (TKIs) changed dramatically the prognosis of CML patients, turning a once fatal disease, into a chronic and manageable disorder. Analysis of CML patients treated with TKIs revealed a potential correlation between toxic effects of TKIs and levels of ROS.

Even though the innovation of this therapy has significantly improved the life expectancy CML patients, still, in some cases, this treatment becomes ineffective.

In order to clarify these observations, we measured and correlated the level of oxidative stress between healthy individuals and patients with chronic myeloid leukemia (CML) treated with first generation tyrosine kinase inhibitors (TKIs).

The two markers of oxidative stress, d-ROMs and OSI were significantly higher in the group of patients with CML compared to healthy subjects. No statistically significant differences were observed between CML patients and healthy subjects regarding the oxidative stress marker PAT.

Key words: reactive oxygen radicals (ROS), chronic myeloid leukemia (CML), oxidative stress (OS), imatinib, d-ROMs, PAT, OSI.

Introduction

Reactive oxygen species (ROS) are oxygen containing radicals competent of independent existence with one or more unpaired electrons, and are a byproduct of normal metabolism of oxygen through various enzymatic pathways.

Free radicals,both ROS and reactive nitrogen species (RNS) can be generated from endogenous or exogenous sources.Immune cell activation, inflammation, ischemia, infection, cancer, excessive exercise, mental stress, and aging are all responsible for endogenous free radical production [1].

There are multiple external triggers that along with endogenous ROS sources can promote oxidative stress, such as air pollutants, heavy metals (Cd, Hg, Pb, Fe, and As), smoking, ionizing and nonionizing radiations, certain drugs (cyclosporine,tacrolimus,gentamycin,), cooking (smoked meat, used oil, and fat), alcohol, pesticides, chemical agents like quinones, organic solvents etc.[2].

Oxidative stress (OS) is defined as imbalance between ROS production and antioxidative defense mechanisms, thus disruption of the cellular redox status either through excessive production of ROS or through inadequate antioxidant response of the cells can result in OS.

While low or moderate levels of ROS/RNS are beneficial and involved in various physiological cell functions such as in immune function, in the proper function of many signaling cell pathways and in the regulation of cell redox status, higher concentrations of both ROS and RNS lead to oxidative and nitrosative stress respectively, which can finally result in cell damage through different mechanisms[3].

For instance, an excess of hydroxyl radical and peroxynitrite can cause lipid peroxidation, thus damaging cell membranes and lipoproteins.Being a radical chain reaction,lipid peroxidation spreads very quickly and it affects a large amount of lipid molecules. Proteins can also be damaged by oxidative stress because ofconformational modifications that could lead to loss or impairment of their enzymatic activity.Several enzymes are crucial against fighting oxidative stress, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase and are known as endogenous protein antioxidants.

SOD exists in three different isoforms: SOD1, SOD2, and SOD3. SOD1 is distributed throughout the cell cytoplasm, cell nucleus, and in the lumen between outer and inner membranes of mitochondria. SOD2 isoforms are located in the matrix of mitochondria, while SOD3 is found mostly extracellularly. SOD is an enzymatic antioxidant that catalyzes the conversion of $O_2^-$to H_2O_2 and helps maintain the redox balance by diffusing the superoxide [4].

Medicinally, increasing the levels of SOD could be an important treatment strategy in oxidative stress-induced pathology. A lot of clinical researches have shown that a lot of neurodegenerative diseases are highly connected with changes in the function of this enzyme such as amyotrophic lateral sclerosis (ALS).

SOD2 is considered to be one of the most important antioxidant components of the cells. It is a homotetrameric enzyme and requires a manganese as its active center. MnSOD constitutes about 10-15% of the total SODs and is localized in the mitochondria of type II pneumocytes, alveolar macrophages, and bronchial epithelial cells of human lungs. MnSOD mRNA is predominantly expressed in cells of the airway walls, the septal tips of alveolar ducts and in arteriolar walls located adjacent to airways.

MnSOD is induced by altered cellular redox states, inflammatory cytokines such as interleukin-1 and unterleukin-6, interferon, tumor necrosis factor alpha and cigarrete smoke. SOD3 is a secretory extracellular enzyme located in the interstitial matrix of tissues such as lung, blood vessels, kidneys, uterus, and to a lesser extent in heart, and responsible for the maintenance of redox homeostasis and matrix components of such tissues.

SOD3 is well known ho nave not only free radical scavenging properties but also angiogenic, anti-inflammatory, anti-chemotactic and anti-proliferative properties.

Catalase (CAT) is the most widespread enzyme in humans.CAT is found in most aerobics organisms but also in anaerobic bacteria. CAT shows activity in the brain, heart, skeletal muscles, spleen, yet the highest activity of this enzyme is in the erythrocytes and the liver.Inside the cells it is found in peroxisomes, where it occurs in free form and bound to the membrane and in mitochondria [5].

In erythrocytes almost the entire amount of this enzyme is found in the cytosol.As for the participation of catalase in the origin and development of diseases, it has been shown that this enzyme has an important role in the molecular mechanisms of inflammation, mutagenesis, apoptosis, and tumorogenesis. Alterations in its activity have been detected in patients with diabetes, malnutrition, hemolytic anemia, liver failure, pancreatitis, muscular dystrophy and neonatal sepsis.

Glutathione peroxidase (GPx) is a selenocysteine-dependent protein. Selenium is the active center of the enzyme. It is located on the very surface of the enzyme and is accessible for binding with the substrate, which enables a high rate of enzyme reaction.In almost all cells this enzyme is found in mitochondria, cytosol, peroxisomes and the intermembrane space.

This enzyme has significant role in preventing the phase initiation of lipid peroxidation.

Beside these, there are exogenous antioxidants which enter the organism through the diet (existing in determined foods) such as Vitamins (Vitamin C, Vitamin E, Vitamin A), flavonoids or through supplements with antioxidant formulations, and co-factors (copper, zinc, manganese, iron, and selenium).

Vitamin C also known as ascorbic acid is an antioxidant hydrosoluble vitamin, this due to that it is an electron donor, which explains its being a reducer that directly neutralizes or reduces the damage exercised by electronically disequilibrated and instable reactive species, denominated

Free radicals (FR). The presence of this vitamin is required for a certain number of metabolic reactions in all animals and plants and is created internally by nearly all organisms, humans comprising a notable exception [6].

Vitamin C is essential for the biosynthesis of collagen proteins, carnitine (which is a pro-catabolic transporter of fatty acids in the mitochondria), neurotransmitters (cell communication mediators, primarily of nerve expression), neuroendocrine peptides, and in the control of angiogenesis, it aids in the development of teeth and gums, bone, cartilage, iron absorption, metabolism of fats, it promotes resistance to infections and repair of normal connective tissue.

Vitamin E is a group of methylated phenolic compounds which are known as tocopherols. Alpha-tocopherol is the most common of these and biologically that with the greatest vitaminic action [7].

It is a lipophilic antioxidant that is localized in the cell membranes whose absorption and transport are found to be very highly linked with that of the lipids. It is considered the most important lipid molecule protector because its action consists of protecting the polyunsaturated fatty acids of cell membrane phospholipids from cellular peroxidation and also it inhibits the peroxidation of low-density lipoproteins.

Tocopherols protect the highly stored fatty acids that are present in cellular and subcellular membranes, maintaining the integrity of the biological membranes from the oxidative damage that they could undergo on acting as free radical traps [8].

It has also been suggested that the tocopherols play an important role in cell respiration and in DNA and co-enzyme biosynthesis.

Tocopherols show anticoagulant activity because of their ability to inhibit platelet hyperaggregation, and to reduce production of prostaglandins, suggesting their role in prevention of atherosclerosis, and thrombotic events. Also, they are known for helping the recycling of vitamin C, protection against prostate cancer, and for their potent anti-aging properties.

Vitamin A belongs to a family of liposoluble compounds that are essential in the diet. It is an antioxidant vitamin that eliminates free radicals (FR) and inhibits DNA mutagenesis. Vitamin A is important for proper vision, normal growth (its deficiency causes bone growth delay), reproduction, cellular proliferation, spermatogenesis, and fetal development [9].

By removing reactive oxygen, hydroxyl radicals, hydroperoxides, and lipid peroxides, flavonoids show various anti-inflammatory, antimicrobial, antithrombotic, anticancer, and antioxidant properties [10].

As it is stated above, free radicals damage all cell molecules, causing breakage of intermolecular bonds, reduction of the fluidity and permeability of cell membranes, that finally leads to cell death. The damage of proteins, lipids and DNA is an important basis of many diseases such as atherosclerosis, neurodegenerative, metabolic, and chronic inflammatory diseases, and cancer.

Chronic oxidative stress has harmful effects throughout the multistage process of carcinogenesis, causing DNA damage, altered DNA repair, epigenetic changes, altered apoptosis, and disruption of signal transduction responsible for maintaining normal cellular homeostasis, angiogenesis and metastasis [11]. Excessive reactive oxygen species production in carcinomas has been shown to induce various biological

effects includingincreasement of cell proliferation, DNA damage and instability, cell damage and death, autophagy and drug resistance [12].

Oxidative stress is involved in all three phases of the complex process of carcinogenesis[13].

It is proven that OS is involved in the pathogenesis of chronic myeloid leukemia (CML), a myeloproliferative neoplasm characterized by uncontrolled proliferation of maturing and mature myeloid cells, caused by the presence of translocation t(9;22) leading to the abnormal BCR-ABL fusion protein. Historically treatment options for this disease were limited to hydroxyurea, busulfan, and interferon-α (IFN-α), with allogeneic stem cell transplant being the only potential curative therapy, but still with poor prognosis [14].

Fortunately, the introduction of tyrosine kinase inhibitors (TKIs) changed dramatically the prognosis of CML patients, turning a once fatal disease, into a chronic and manageable disorder. Analysis of CML patients treated with TKIs revealed a potential correlation between toxic effects of TKIs and levels of ROS. Even though the innovation of this therapy has significantly improved life expectancy CML patients, still, in some cases, this treatment becomes ineffective.

Two types of TKI resistance have been described, primary and secondary, and many studies have evaluated the involvement of oxidative stress in this setting of patients [15].

In order to clarify these observations, we measured and correlated the level of oxidative stress between healthy individuals and patients with chronic myeloid leukemia (CML) treated with first generation tyrosine kinase inhibitors (TKIs).

Material and methods

The open, single center clinical study was performed on 25 patients of both sexes (15 male and 10 female) with chronic myeloid leukemia who are already on Imatinib therapy and a control group consisting of 24 healthy subjects (12 male and 12 female).

Inclusion criteria for patients with chronic myeloid leukemia and healthy subjects were:

- male and female patients and healthy subjects aged ≥ 18 years;
- patients with diagnosed chronic myeloid leukemia receiving imatinib for longer than 1 month;
- patients and healthy subjects who at the time of inclusion did not received antioxidants more than 15 days;
- patients and healthy subjects who agreed to participate in the research and signed an informed consent.
- able to communicate and co-operate with the investigator and his staff;
- capable of consent and sign an informed consent.

The patients were diagnosed with chronic myeloid leukemia at University Clinic of Hematology, Medical faculty, ss. Cyril and Methodius, Skopje, R.N Macedonia and received Imatinib therapy longer than 1 month.

Healthy subjects were recruited at Institute of Preclinical and Clinical Pharmacology and Toxicology, Medical faculty, ss.Cyril and Methodius, Skopje, N.Macedonia. The healthy subjects were in good health as determined by the medical and medication history, complete physical examination(including vital signs), ECG and clinical laboratory tests (blood count, biochemical profile, and urinalysis).

Method for determination of d-ROMs, PAT and Oxidative stress index

PAT (total antioxidant power, iron reducing) and d-ROMs (plasma peroxides) were measured on FRAS5 analytical photometric system by using REDOX fast kit (made of 50 individual d-ROMs fast tests

and 50 individual PAT tests, H&D srl, 43124 Parma, Italy), upgraded by H&D srl but initially developed by Mauro Carratelli [16,17].

All samples were stored frozen in an upright position in freezers at the Institute of preclinical and clinical pharmacology and toxicology at a temperature -80 ± 10ºC until analyzed.The procedure was done according to the producers' guidelines for the both, d-ROMs and PAT tests.

The d-ROMs and PAT are reported in equivalents of H_20_2 and ascorbic acid, respectively.

The d-ROMs and PAT reference normal values are 250 – 300 U. Carr (1 U. Carr = 0.08 mg H_20_2/dL) and 2200 – 2800 U. Carr, respectively.

Oxidative stress index (OSI) presents information obtained from d-ROMs fast test and the PAT test that is automatically calculated by the dedicated spectrophotometer FRAS5 with normal reference values less than 40 given by the manufacturer (H&D srl, 43124 Parma, Italy).

Statistical Method

Data points had been plotted in Microsoft Excel and statistical analysis of the data was performed with Student's t-test. Statistical significance, was considered, in all instances for $p<0.05$. All data have been reported as average±standard deviation, minimum and maximum.

Results

The results of the values obtained for the determined markers of oxidative stress (d-ROMs, PAT and Oxidative stress index) in male and female patients with chronic myeloid leukemia treated for longer than 1 month with imatinib and in male and female healthy subjects are shown in table 1 and 2.

Table 1. Oxidative stress markers (d-ROMs, PAT and Oxidative stress index) in patients with chronic myeloid leukemia treated with imatinib (male and female).

	d-ROMs	**PAT**	**OSI**
Average	395,08	2780,80	83,52
±SD	74,21	707,54	22,62
Minimum	236	1702	27
Maximum	488	4818	121
N	25	25	25

Table 2. Oxidative stress markers (d-ROMs, PAT and Oxidative stress index) in healthy subjects (male and female).

	d-ROMs	**PAT**	**OSI**
Average	262,50	2571,21	39,67
±SD	44,35	436,07	18,99
Minimum	147	1918	8
Maximum	343	3594	77
N	24	24	24

The results of the values obtained for the determined markers of oxidative stress (d-ROMs, PAT and Oxidative stress index) in female patients with chronic myeloid leukemia treated for longer than 1 month with imatinib and and in female healthy subjectsare shown in table 3 and 4.

Table 3. Oxidative stress markers (d-ROMs, PAT and Oxidative stress index) in female patients with chronic myeloid leukemia treated with imatinib.

	d-ROMs	**PAT**	**OSI**
Average	402	2787	88,90
±SD	83,88	764,62	21,01
Minimum	236	1702	60
Maximum	488	4326	121
N	10	10	10

Table 4. Oxidative stress markers (d-ROMs, PAT and Oxidative stress index) in female healthy subjects.

	d-ROMs	**PAT**	**OSI**
Average	257,42	2376,33	44,92
±SD	57,14	279,22	15,76
Minimum	147	1918	20
Maximum	343	2868	73
N	12	12	12

The results of the values obtained for the determined markers of oxidative stress (d-ROMs, PAT and Oxidative stress index) in male patients with chronic myeloid leukemia treated for longer than 1 month with imatinib and in male healthy subjects are shown in table 5 and 6.

Table 5. Oxidative stress markers (d-ROMs, PAT and Oxidative stress index) in male patients with chronic myeloid leukemia treated with imatinib.

	d-ROMs	**PAT**	**OSI**
Average	390,47	2776,67	79,93
±SD	69,72	694,47	23,61
Minimum	239	1890	27
Maximum	459	4818	104
N	15	15	15

Table 6. Oxidative stress markers (d-ROMs, PAT and Oxidative stress index) in male healthz subjects treated with imatinib.

	d-ROMs	**PAT**	**OSI**
Average	267,58	2766,08	34,42
±SD	28,13	486,60	21,12
Minimum	230	2147	8
Maximum	311	3594	77
N	12	12	12

Figure 1, 2 and 3 show the results obtained for markers of oxidative stress (d-ROMs, PAT and Oxidative stress index) in patients with chronic myeloid leukemiatreated for more than 1 month with imatinib and in healthy subjects.

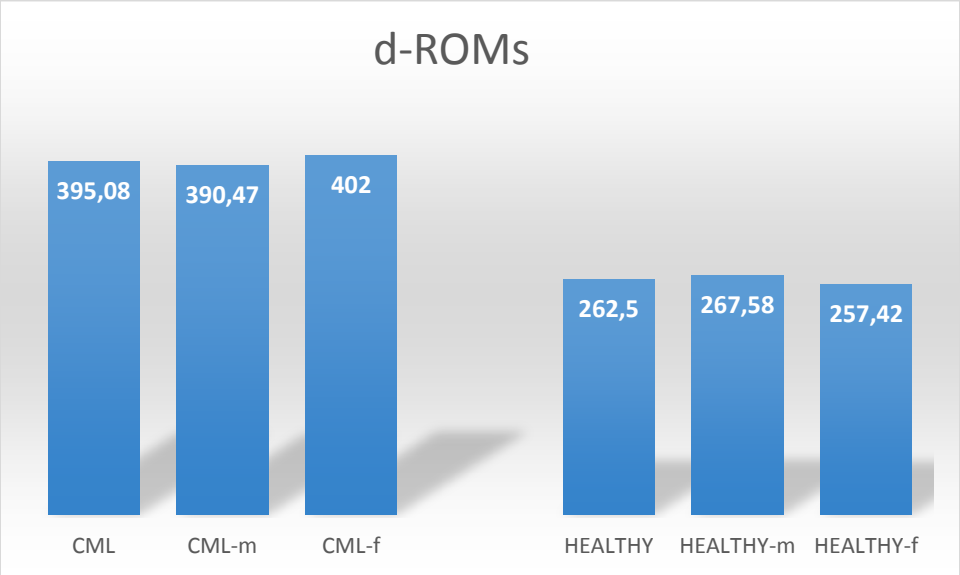

Figure 1. Oxidative stress markers-d-ROM in patients with chronic myeloid leukemia treated with imatinib (all, male, and female) and in healthy subjects (all, male, and female).

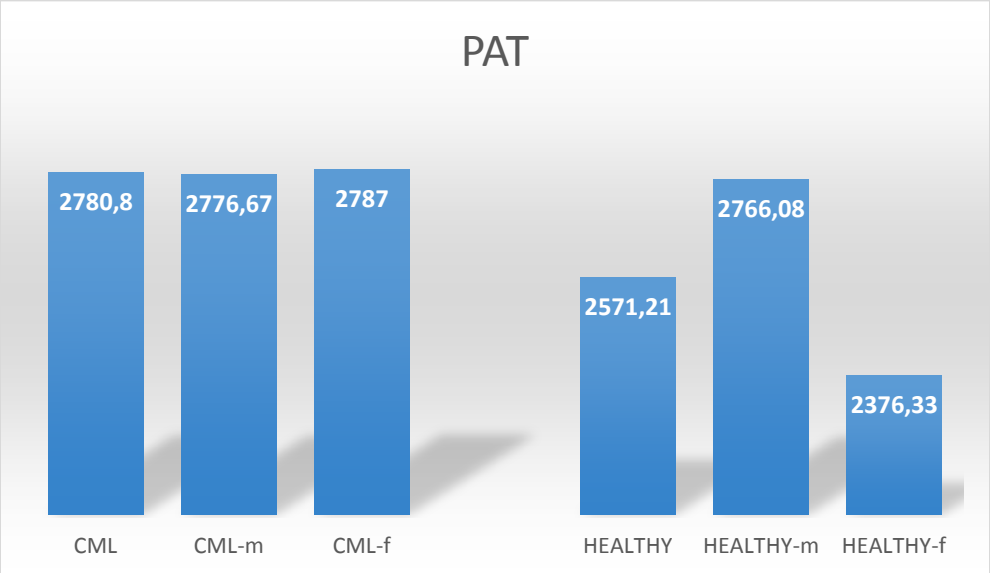

Figure 2. Oxidative stress marker-PAT in patients with chronic myeloid leukemia treated with imatinib (all, male, and female) and in healthy subjects (all, male, and female) :

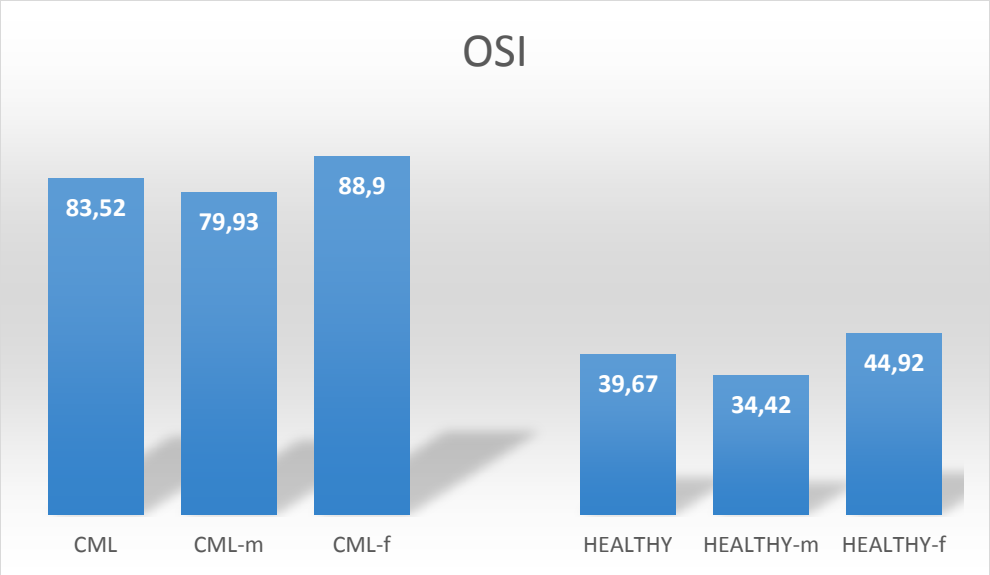

Figure 3. Oxidative stress marker-OSI in patients with chronic myeloid leukemia treated with imatinib (all, male, and female) and in healthy subjects (all, male, and female) :

The biggest interindividual differences were observed for the oxidative stress marker-OSI (Oxidative stress index), while the other two markers of oxidative stress (d-ROMs and PAT) did not show large interindividual differences.

The values obtained for the markers of oxidative stress-d-ROMs and Oxidative stress index (OSI) are higher in the group of patients with chronic myeloid leukemia treated for longer than 1 month with imatinib compared to healthy subjects by 50.5% for the marker d-ROMs and 210% for Oxidative stress index.

The statistical analysis of the obtained results for the oxidative stress markers (d-ROMs and Oxidative stress index) in the patient group and in the healthy subjects showed that there is a highly statistically significant difference in terms of d-ROMs and OSI between the patient group and the healthy subjects (Student's t-test, $p<0.001$). The values for d-ROMs and OSI index are significantly lower in the group of healthy subjects compared to the group of patients.

The statistical analysis by gender (female and male) between the two groups for the two mentioned markers showed that there are highly statistically significant differences between the two groups and for both markers and for both genders (Student's-t-test, $p<0.001$).

The marker of oxidative stress-PAT (total antioxidant power) did not show a statistically significant difference between the group of patients with chronic myeloid leukemia treated for longer than 1 month with imatinib and healthy subjects (Student's-t-test, $p >0.05$), although the obtained mean values are higher in the patient group by 8.1% compared to healthy subjects.

The statistical analysis by gender for the marker of oxidative stress-PAT between the two groups of subjects showed that there are no statistically significant differences between the two compared groups (Student's-t-test, $p>0.05$).

No statistically significant differences were found in relation to the three markers of oxidative stress (d-ROMs, PAT and OSI when comparing the group of male patients with the group of female patients (Student's-t-test, $p>0.05$), as well as when comparing the group of healthy male subjects with the group of healthy female subjects (Student's-t-test, $p>0.05$).

Discussion

Published data in the literature so far show that oxidative stress is involved in the pathophysiology of malignant diseases and that it plays a role in the initiation and progression of the disease, as well as in the development of resistance to some drugs. There is evidence that the proapoptotic effect of oxidative stress is induced by some oncological therapies.

In patients with chronic myeloid leukemia, segments of the long arms of chromosome 9 and chromosome 22 break and separate. Then the places between the segments of the two chromosomes are exchanged and the separated segment from chromosome 9 goes to chromosome 22 and vice versa. In this way, an unnaturally long chromosome 9 and an unnaturally short chromosome 22 are formed.

The altered chromosome 22 in this way is called the Philadelphia chromosome. The separated segment of chromosome 9 where the ABL1 gene is located fuses with chromosome 22 at the site where the BCR gene is located and a new gene is formed called BCR-ABL1.

This gene codes for the synthesis of a protein that is constantly active and acts on other proteins that control the cell cycle and accelerate cell division and prevent DNA regeneration, thus allowing the creation of other mutations and abnormalities. The BCR-ABL gene also causes excessive tyrosine kinase activity that leads to pathological, unregulated proliferation of myelocytes [18,19].

In chronic myeloid leukemia, there is evidence that oxidative stress is involved in the initiation of the genetic mutation responsible for the formation of the BCR-ABL oncogene, and the induced oxidative stress leads to disease progression, additional mutations and the development of resistance to drugs [20-23].

Imatinib is a synthetic tyrosine kinase inhibitor used in the treatment of chronic myeloid leukemia. Various mechanisms of imatinib resistance have been identified, including BCR-ABL gene overexpression, BCR-ABL kinase mutations, and genetic variation and/or altered expression of imatinib genetic transporters. Among them, reactivation of the BCR-ABL protein by mutations in the kinase domain, such as the T315I mutation, has been documented as one of the most prevalent mechanisms leading to imatinib resistance.

ABL protein by reactive oxidative compounds (ROS) can lead to a change in the three-dimensional structure of the ABL kinase domain of this oncoprotein which is the site of action of imatinib. Research in recent years has attributed a greater role in the development of resistance to imatinib to oxidative stress.

The impact of oxidative stress in patients with chronic myeloid leukemia is still unclear. Previous studies have shown that the transformation of the BCR-ABL oncogene can promote the generation of reactive oxidative compounds and redox imbalance. It is well known that excessive production of reactive oxidative compounds such as superoxide radicals ($O2^-$), hydrogen peroxide (H2O2) and highly reactive hydroxyl radicals (OH^-) leads to oxidative damage to lipids, proteins and DNA. If not corrected , DNA damage can lead to mutagenesis and cause the initiation or development of various types of malignant tumors.

Our results confirm that in chronic myeloid leukemia patients treated with imatinib there is a significantly higher level of oxidative stress compared to healthy subjects as measured by markers of oxidative stress, d-ROM and oxidative stress index (OSI).

Patients with chronic myeloid leukemia who were participants in our trial were on imatinib therapy longer than 1 month (6 to 74 months), and due to it is not possible to conclude how much of the oxidative stress is due to the disease itself, and how much of imatinib treatment. To obtain this data, it is necessary to determine the markers of oxidative stress in a sufficient number of patients with chronic myeloid leukemia who have not started therapy with imatinib.

The absence of significant differences between the group of patients and the group of healthy subjects regarding the marker of oxidative stress-PAT is probably due to the fact that the endogenous antioxidant capacity of the organism is not fully used, and it is possible to the lack of complete and accurate data on the nutritional status. and the use of supplements as adjuvant therapy at the time of blood sampling [24].

Conclusions

Patients with chronic myeloid leukemia treated with imatinib had significantly higher level of oxidative stress compared to healthy subjects as determined by the oxidative stress markers d-ROM and the oxidative stress index (OSI), but not by the oxidative stress marker-PAT Due to the insufficient number of patients who had not received imatinib before the blood sampling, we could not determine the influence of imatinib on the level of oxidative stress in patients with chronic myeloid leukemia.

It can be assumed with a high level of certainty that the determined higher level of oxidative stress in patients with chronic myeloid leukemia treated with imatinib for longer than one month may have an impact on the development of resistance to the drug.

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