

PRELIMINARY STUDY OF CELL METABOLISM, BY USE OF NBT TEST, DETERMINATION THE INTENSITY OF LIPID PEROXIDATION AND ANTIOXIDANT ACTIVITY

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Abstract. Otto Warburg, in the early part of the 20th century, originated a hypothesis, that the cause of cancer is primarily a defect in energy metabolism.

A decrease in the capacity of mitochondria to reduce NAD(P), together with a decline in the NAD(P)H/NAD(P) redox couple, uncouples oxidative phosphorylation, lead to depletion of ATP and decrease the cell viability.

Nitro-bleu tetrazolium have been used to assay cell proliferation and viability. The method to measure cell proliferation is based on enzymatic cleavage of the tetrazolium salts to a water-soluble formazan dye.

Succinate-tetrazolium reductase, is an enzymatic system, which belongs to the respiratory chain of the mitochondria and it is active only in viable cells. The reagent diffuses into the cells and it is cleaved to formazan. The absorption change is measured and analysed.

Free radicals such as superoxide, can cause a damage in cellular components, but several antioxidants inhibiting the lipid peroxidation and limiting the level of free radicals in cells.

In the present study we had in view the proliferation and viability of leukemia cells during antineoplastic treatment along with the alteration of the serum level of malondialdehyde (MDA) and ceruloplasmin (CP). With serum level of malondialdehyde we monitored the presence of the lipid peroxidation by the reactive oxygen species, and with the oxidized ceruloplasmin level in blood serum we evidenced the activity of antioxidant system in blood.

Keywords: nitro-bleu tetrazolium, leukemia, cell proliferation, MDA, CP

INTRODUCTION

In the early part of the 20th century, Otto Warburg originated a hypothesis that the cause of cancer is primarily a defect in energy metabolism. This hypothesis was based on the observation, since repeated and verified many times, that cancer cells show clear differences in energy metabolism when compared to normal cells [12].

In the respiratory chain, during transfer of electrons to molecular oxygen, an estimated 1 to 5% of electrons lose their way, most participating in formation of reactive oxygen species [3].

However, generation of reactive oxygen species (ROS), may be a relatively late event, occurring after cells have embarked on a process of caspase activation. A decrease in the capacity of mitochondria to reduce NAD(P), together with a decline in the NAD(P)H/NAD(P) redox couple, permeabilizes the inner mitochondrial membrane. This favors the release of Ca^{2+} from the organelle and uncouples oxidative phosphorylation and these effects lead to depletion of ATP [13].

The reduced ATP levels also lead to further impairment of other Ca^{2+} regulation system, in the plasma membrane and the endoplasmic reticulum. In addition, the decrease in NAD(P)H compromises the activity of protective enzymes, which further increases the deleterious effects of ROS [6, 12].

Cancer cells exhibit unlimited proliferative potential, resistance to cell death stimuli and abnormal energy metabolism [7].

Several tetrazolium salts, including NBT (nitro bleu tetrazolium) have been used to assay cell proliferation and viability.

Succinate-tetrazolium reductase, is an enzymatic system, which belongs to the respiratory chain of the

mitochondria and it is active only in viable cells [19, 12].

The total activity of this mitochondrial dehydrogenase increases proportionally to the number of viable cells, leading to an increase in tetrazolium salt conversion to formazan dye, which is in turn quantified by absorbance. We can analyse cytotoxic and cytostatic compounds as anticancer drugs and other pharmaceuticals.

During the respiratory explosion, the increase of the oxygen metabolism and the production of ROS is accompanied by the increase of the reduction potential in phagolysosomes and the turning into blue of NBT [16].

The more the cellular metabolic activity is intensified, and consequently the oxidation reduction reactions take place at a higher intensity, the more a larger quantity of formazan will result, colouring the cells more intensely. In the more of the cell displays a decreased metabolic activity, or it is dead, the smaller the amount of formazan resulted.

The exogenous cellular stresses, such as chemotherapy or increased level of reactive oxygen species (ROS) are activators to mitochondrial disruption [5].

Free radicals such as superoxide and hydroxyl radicals, can cause a damage in cellular components via peroxidation of proteins, nucleic acids, free amino acids, and lipoproteins. Free radicals first attack fatty acids, because the C=C double bonds in these molecules are sensitive to oxidative damage [9].

In a cell, where free oxygen radicals accumulate, the fatty acids, which would normally be subjected to beta-oxidation within the mitochondria, will be subjected to lipid peroxidation, production of toxic and reactive aldehyde metabolites such as malondialdehyde

(MDA) which is commonly used as an index of lipid peroxidation [8, 14].

Ceruloplasmin (CP) is an enzyme, which have antioxidant activity, inhibiting the lipid peroxidation and limiting the level of free radicals in cells. The oxidized ceruloplasmin level in blood serum is evidence the activity of antioxidant system in blood [16].

In the present study we had in view the proliferation and viability of leukemia cells during antineoplastic treatment along with the alteration of the seric level of malondialdehyde and ceruloplasmin.

MATERIALS AND METHODS

It have been studied leukemia cells. We were monitored in various stages of the antineoplastic treatment, the cell proliferation and cell viability by use of nitro bleu tetrazolium, the serum level of malondialdehyde and ceruloplasmin.

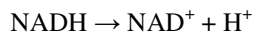
Blood samples were taken from the patients before the treatment and after 24 after the beginning of polychemotherapy.

NBT test

The formazan salt (NBT) diffuses into the cell and it is cleaved to formazan, by tetrazolium succinate reductase, a system belonging to the mitochondrial respiratory chain and active in the viable cells.

NBT is turned into formazan by breaking the N-N bonds within the molecule, and the N adds a H⁺ atom, representing an intermediary electron acceptor.

H⁺ results from NADH, which will thus be oxidized:



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Materials needed

-Test tubes with Na₂EDTA, used for blood sample taking;

-NBT (1%) stock solution, prepared from 10 mg NBT and 1 ml bidistilled water. The mixture obtained this way can be kept in the refrigerator at 4 °C for several months;

-Michaelis buffer, pH 7.4, prepared from Na hydrochlorate 0.85 % neuter solution; 5 ml veronal acetate solution; 5 ml hydrochloric acid N/10;

-Methylic alcohol [4, 5].

Working method

1-2 ml blood is drawn into a test tube containing 2 mg Na₂EDTA and shaken. 0.08 ml Michaelis buffer pH 7.8 is added into the test tube; 0.02 ml 1% NBT solution. 0.10 ml 0.2 % NBT solution is obtained this way. 0.10 ml blood drawn on Na₂EDTA is added.

The test tube is incubated in the thermostat, at 37⁰C, for 30 minutes. The numeration of cells it realized at microscope. The cells which are colored in blue have formazan sediment. With numeration of this cells we can determination the rate of cell viability and cell proliferation. When the percentage of viable cells are below 30% it considered the NBT test negative, but when above 30%, it considered the NBT test positive.

Colorimetric determination of malondialdehyde

Malondialdehyde is one of the products of lipid peroxidation, it's determination represents a standard method of assessing the oxidative stress.

The dosage method is based on the reaction with thiobarbituric acid (TBA). The biological sample is heated with TBA, in acidic medium. As a result of the reaction, one molecule of MDA reacts with two molecules of TBA, with the production of a pink pigment, with a measured optical density at 530 nm, using Pharmacia LKB Ultraspec. III spectrophotometer.

Normal values of the MDA serum levels are between 0.27 – 1.02 nmol/ml. Increased values of the MDA serum levels confirm the presence of the oxidative stress [11].

Colorimetric determination of ceruloplasmin

Ceruloplasmin (ferroxidase) is a 150 kD, blue α₂-glycoprotein that is synthesized in the liver and it is accumulated in the matrix and the inner membrane of the mitochondria. Ceruloplasmin acts mainly as a ferroxidase, catalyzing the oxidation of Fe (II) to Fe (III), and as a Fe (II) carrier in the plasma in association with transferrin, the only protein which can carry iron in this state.

Beside its detoxifying activity in the blood, ceruloplasmin also presents a dismutase-like activity (lower than that of the superoxide dismutase), it inhibits the peroxidation of polyunsaturated fatty acids (in vitro demonstration) and it has immunologic activity. Ceruloplasmin limits the quantity of free radicals, acting as a plasmatic antioxidant [9, 15].

Normal ceruloplasmin serum levels are between 0.20-0.40 mg/ml (11-24 μM).

Abnormal ceruloplasmin level impedes the mitochondrial respiratory process [1, 16].

The principle of the method is based on the phenol oxidative property of ceruloplasmine, which catalyzes the oxidation of paraphenylenediamine, with the production of a violet compound.

The optical density against a reference blank was measured at 530 nm, using Pharmacia LKB. Ultraspec. III spectrophotometer. The ceruloplasmine quantity in 1 ml can be determined using the formula:

$$E \times 0.87 = \text{CP serum level (mg/ml)}$$

Abnormal ceruloplasmine level impedes the mitochondrial respiratory process. The ceruloplasmine serum level in patients under polychemotherapeutic treatment was monitored in order to determine the presence of plasmatic antioxidant during the antineoplastic treatment [16].

RESULTS

In Figure 1 and 2 we are showing two examples of NBT test before and after beginning the antineoplastic treatment.

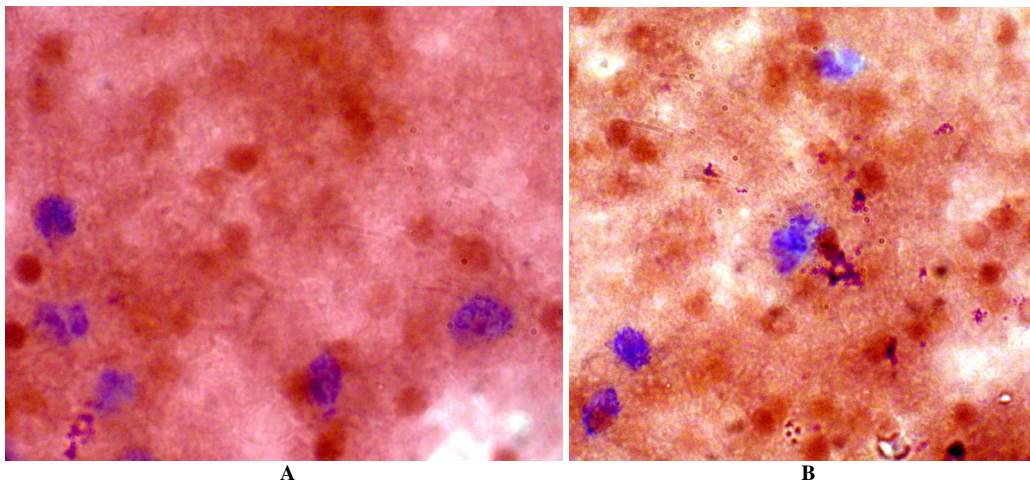


Figure 1. Positive NBT test, when **A** represent NBT test from blood derived by subject 1 before treatment and **B** represent NBT test from blood derived by subject 2 before treatment (400X).

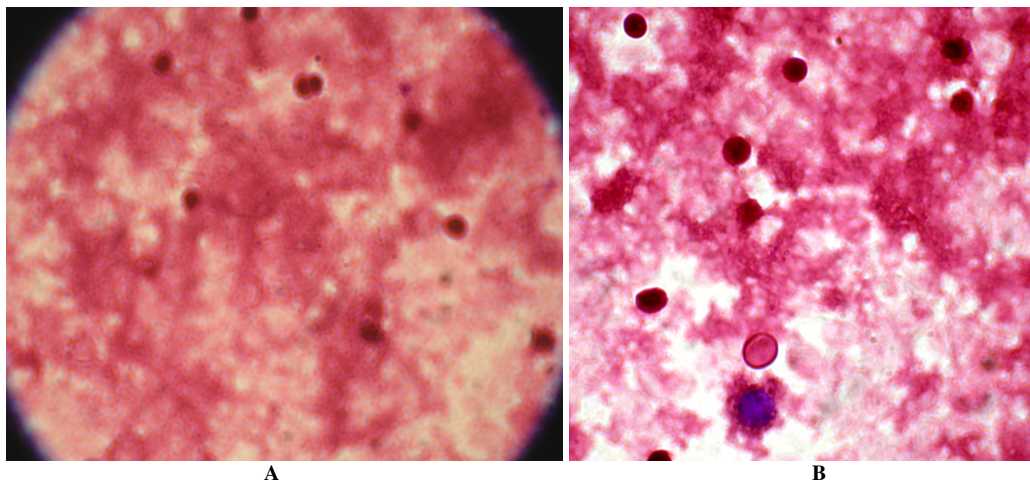


Figure 2. Negative NBT test, when **A** represent NBT test from blood derived by subject 1 after 24 hours at the beginning of treatment and **B** represent NBT test from blood derived by subject 2 after 24 hours at the beginning of treatment (400X)

We can see in Figure 1 that before the antineoplastic treatment the cells display proliferation, they are alive. This proliferation significantly decreases after 24 hours of treatment (Fig. 2), the cells displays a decreased

metabolic activity, together with a decline in the NAD(P)H/NAD(P) redox couple, resulted the smaller amount of formazan.

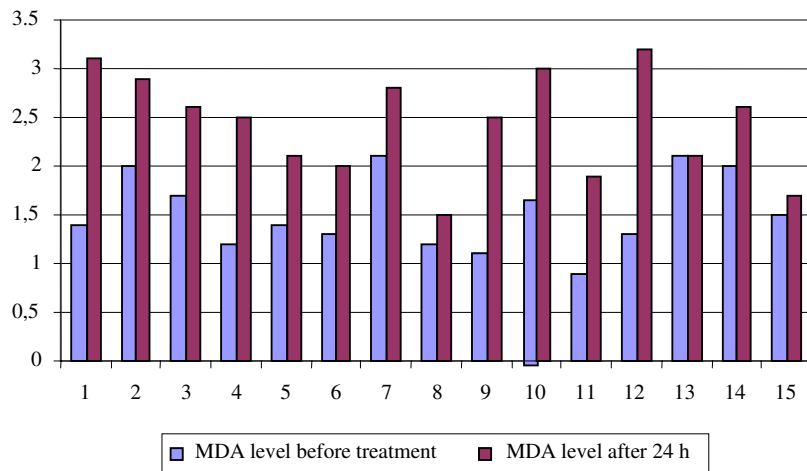


Figure 3. Serum level of MDA before treatment and after 24 hours

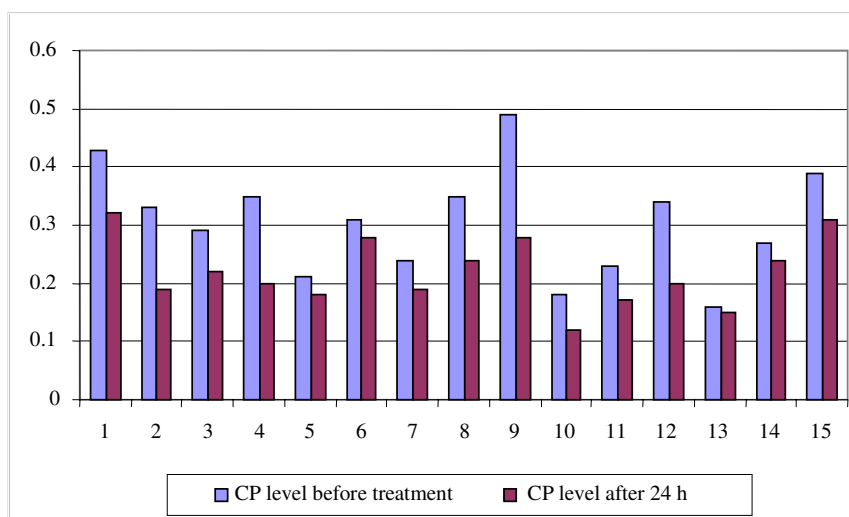


Figure 4. CP serum level before antineoplastic treatment and after 24 hours

In Figure 3 we are showing the serum level of MDA before treatment and after 24 hours. In Figure 4 we can observe that the serum level of ceruloplasmin decreased after 24 hours after the beginning of treatment.

The cytotoxicity of the antineoplastic agents is manifested by the increase in the level of free radicals, by the peroxidation of polyunsaturated fatty acids at mitochondrial level, this occurring without an immediate response of the organism, which would be manifested by an increase in the ceruloplasmin level.

DISCUSSIONS

Cancer cells exhibit unlimited proliferative potential, resistance to cell death stimuli and abnormal energy metabolism. This metabolic alteration has been observed in many cancer types, including leukemia [7].

It is now recognized that the Warburg effect (respiration chain deficient) represents a prominent metabolic characteristic of malignant cells.

An efficient chemotherapy depends on the induction of the appearance of some modifications on the signal ways of the programmed death of cells [2].

Before the treatment the cells display proliferation, they are alive. This proliferation significantly decreases after 24 hours of treatment, the cells display a decreased metabolic activity, together with a decline in the NAD(P)H/NAD(P) redox couple.

The presence of the oxidative stress, of the accumulation of the reactive oxygen species, due to lipid peroxidation is confirmed throughout the study.

Increased level of ROS (reactive oxygen species) in cells cause mitochondrial dysfunction, the dysfunction of the mitochondrial respiratory chain, which can induce a more efficient response to antileukemic therapy through beginning the apoptosis process [3].

The ceruloplasmin serum level in patients under polychemotherapeutic treatment was monitored in order to determine the presence of plasmatic antioxidant during the antineoplastic treatment, as well as to indicate a possible mitochondrial disorder, because of too low ceruloplasmin serum levels. The

oxidative activity of ceruloplasmin towards paraphenylenediamine is used for dosing the oxidative activity, therefore for dosing ceruloplasmin [11].

The cytotoxicity of the antineoplastic agents is manifested by the increase in the level of free radicals, by the peroxidation of polyunsaturated fatty acids at the mitochondrial level, with no other response of the organism subjected to treatment. The decrease of cell proliferation along with an increased level of the oxidative stress in leukemia cells de

The present study aims to investigate the possible relationship between cell viability, serum malondialdehyde level, an index of lipid peroxidation, and ceruloplasmin level, a protective agent against lipid peroxidation.

A correlation was detected between cell viability and serum level of malondialdehyde. No correlation was detected between the serum malondialdehyde and ceruloplasmin levels. It was suggested that increase in the serum malondialdehyde level might be associated with an imbalance of other antioxidants rather than ceruloplasmin.

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