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EFFECTS OF METHYLPREDNISOLONE AND VITAMIN C THERAPY ON MALONDIALDEHYDE LEVEL IN PATIENTS WITH SYSTEMIC LUPUS ERYTHEMATODES

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Our results suggest that oxidative products of lipid peroxidation have a significant role in the onset and chronic course of systemic lupus erythematodes (SLE). Cellular damage, caused by reactive oxygen species generated due to intensive lipid peroxidation and lowered antioxidative defenses, may be attenuated by corticosteroid drugs. Corticosteroid therapy is one of the possible therapeutic choices for the patients with an inflammatory autoimmune disease. Such an approach enables a large number of patients to avoid the development of the most serious complications of autoimmune diseases (renal failure, ankylosis, premature atherosclerosis). Vitamin C supplementation does not enhance the malondialdehyde concentration lowering effect of methylprednisolone in patients with SLE. *Acta Medica Medianae 2010;49(4):10-15.*

***Key words**: malondialdehyde, oxidative stress, methylprednisolone, vitamin C, lupus erythematodes*

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Introduction

Systemic lupus erythematodes (SLE) is a chronic autoimmune multisystemic disease of an unknown etiology.

The incidence of SLE is 52 per 100.000 people (1). It is more frequent in women, especially in fertile women, approximately 7-11 times more than in men (2). Race, ethnic origin and place of living also affect the disease occurrence. SLE is more often diagnosed in the black than white people, as well as in the inhabitants of more urban and industrial areas than in rural ones.

The pathogenesis of SLE has not been clarified yet. As in other autoimmune diseases, the mechanism of its development is based on three impairments:

1. Autoantibodies' action on unchanged or changed structure of cell surface.

2. Appearance of circulating immune complexes autoantigene-autoantibody leading to tissue damage, and,

3. Activity of sensibilized T-lymphocytes releasing destructive cytokines or cytokines attracting other inflammatory cells to the site of damage (3).

In this case, the main cause is the defective clearance of apoptotic cells (4), leading to secondary necrosis by the release of cells' content and inflammatory mediators. Pathogenic autoantibodies subsequently synthesized are the cause of tissue damage in patients with SLE (5). This inflammatory response is the basic characteristic of SLE. Cytokines, as intercellular signaling molecules, modulate inflammation and immunity and, in such a way, regulate growth, motility and differentiation of leukocytes and other cells that may generate different reactive oxygen species.

Malondialdehyde (MDA) is an organic compound (formula - CH2(CHO)2), with a molecular mass of 72.063g/mol. The enol form of MDA predominates:

$$CH2(CHO)2 \rightarrow HOCH=CH-CHO$$

In organic solvents, cis isomer of MDA is dominant, while in water solvents it is the trans isomer.

Reactive oxygen species (ROS) act on polyunsaturated lipids, leading to MDA formation. MDA is a reactive aldehyde, and one of numerous electrophile species causing cell damage. It is one of the mostly measured end-products of enzymatic and nonenzymatic lipid peroxidation

reactions. Concentration determination of this aldehyde is used as a biomarker of oxidative stress level in the organism, because its concentration in blood and tissues is in correlation with cell damage caused by ROS (6).

In chemical reactions, MDA may function both as nuclephilic and electrophilic compound. Due to its high reactivity, it is mostly bound unspecifically and covalently to various biological molecules (proteins, nucleic acids, phospholipids, etc.) in samples (7). MDA is detectable in proteinic plasma fraction, not in lipoproteinic fraction. Besides, MDA molecules are involved in reactions of self-condensation that results in formation of polymers of different polarity and molecular masses.

MDA may be measured directly in plasma by HPLC (High Pressure Liquid Chromatography) method, based on its characteristic to absorb UV light at 245 nm wavelength (8). Thus, a large number of researchers were unable to detect significant amounts of direct MDA in biological material (9). This is understandable since MDA being formed, reacts quickly, even at extremely low concentrations, with amino and thiol groups (10), or is metabolized in tissues by aldehyde-dehydrogenase (11). MDA is then excreted in urine (12).

Because of various difficulties in measuring MDA by direct methods, in 1970s, indirect methods were introduced. Sato et al. (13) used thiobarbituric acid (TBA) assay for measuring MDA as an index of lipid peroxidation. Later studies showed that color field in TBA assay is the result of numerous lipid peroxidation products, as well as MDA (7). That is the reason why, today, this assay is named TBARS (TBA Reactive Substances) measurement.

Conjugated or polymerized MDA forms hydrolysis in acid solutions, and are thermolabile. Because of this, it is essential that MDA hydrolysis occurs directly in the presence of TBA reagent. Measuring of reactants' concentration with TBA may be performed after protein precipitation (14) or directly in plasma (15). The TBA-MDA conjugate may be detected by spectrophotometry or, after separation, by HPLC or gas chromatography.

It is of extreme importance that, while determining concentrations of reactants with TBA, peroxidation phenomenon is limited in *in vitro* conditions during the heating of incubation mixture. This can be controlled by the use of reagents and materials that do not contain measurable amounts of iron. Antioxidants are the most appropriate for disabling the process of peroxidation *in vitro*, such as butylated hydroxytoluene.

The nonspecificity of condensation reaction must also be taken into consideration – other aldehydes, terminal products of lipid peroxidation (2-alkenals, 2,4-alkadienls), and hydroperoxides, also products of lipid peroxides degradation, may react with TBA and form conjugates (16). All these compounds are colored, but not fluorescent, so fluorimetric MDA measurement is a less sensitive method than spectrophotometry. More specific method for the determination of MDA concentration is HPLC which complies wih the criteria of safety, specificity and sensitivity. It is the method of choice for oxidative stress assessment (7).

Aim

The objectives of this work were to determine:

- MDA concentration as a marker of oxidative stress in patients with SLE;
- MDA concentration as a possible indicator of pharmacotherapeutic success in patients with SLE, after a 3-week therapy with prednisolone;
- MDA concentration as a possible indicator of pharmacotherapeutic success in patients with SLE, after a three-week therapy with prednisolone and vitamin C.

Material and methods

Patients included in the study have been consecutively admitted to the Institute of Rheumatology, Clinical Center Serbia, Belgrade. The patients were male, suffering from SLE, but not from any other chronic disease (liver, bone or endocrine diseases), non-smokers and non-consumers of alcohol.

Groups I and II consisted of patients meeting at least 4 of 11 criteria for SLE posed by American College of Rheumatology (17). The study included 25 male patients of average age 36.5±9.2 years (between 27 and 50 years), having SLE for 5.4±2.7 years. There was no statistically significant difference in age and duration of disease between groups I and II.

Group I was made of 12 patients treated only with methylprednisolone (20 mg, once a day at 8 am). After 3 weeks of treatment, MDA level was assessed.

Group II consisted of 13 patients treated with methylprednisolone (20 mg, once a day at 8 am) and vitamin C (500 mg, once a day at 8 am). After 3 weeks of treatment, MDA concentration was measured.

Patients' serums, used as the analysis samples, were obtained from venous blood, taken after a 12-hour period without food intake, during a regular check-up. Peripheral blood was taken for an analysis by venepunction, and afterwards, serum was obtained by centrifugation at room temperature on 3.500 rotations per minute for 15 minutes. Serums were, then, aliquoted, frozen and held at -80°C until analyses were performed.

Pro-analysis substances were used for reagents preparation. All the reagents were made immediately before the analysis. Into the test tubes containing 0.2 ml of sample, 0.8 ml of

reagent I (50 mmol/l TRIS-HCl, pH 7.4) and 2 ml of reagent II (TBA) were added. Reagent I is being prepared by diluting 1.21 g of TRIS in distilled water, and then HCl is being added until pH 7.4 (at first with concentrated HCl until pH 7.5, and then with 1 mol/l HCl). If necessary, the day after, it may again be needed to adjust pH to 7.4. Reagent II is being prepared, first, by adding 15 ml of 50% TCA to 0.18 g of TBA, and then by mixing it until all TCA is being dissolved. Since the reaction occurs only in acid solution, 1.05 ml of concentrated HCl is added, and then completed to volume of 50 ml with distilled water. Then, the test tubes were closed with glass beads and put into the boiling water bath (at 100°C) for 15 minutes. Other two test tubes (blind probe) contained reagents I and II, but instead of the sample, 0.2 ml of distilled water was added (these test tubes were not incubated at 100°C). After the incubation, test tubes were cooled with tap water, and centrifuged on 3.000 rotations per minute for 10 minutes. After the centrifugation, test tubes were manipulated carefully so the supernatant and the precipitate obtained do not mix. With a micropipette, from each test tube, 200 µl of supernatant was taken for micro-centrifugation.

The samples from test tubes were analyzed in a biochemical analyzer („Evolution 3000", Italy). Since the conjugate MDA:TBA shows the maximal absorption at 536 nm, the analyzer was adjusted with regard to the blind probe, and automatically the absorbance was measured at 536 nm. The method is based on the reaction between TBA and MDA from the sample, one of the end-products of lipid peroxidation (18). At high temperatures and in acid solution, MDA goes into the reaction of nucleophile addition and TBA, and a red-colored conjugate is being produced (reaction ratio is 1 MDA:2 TBA) that has the maximum of absorption of monochromatic light at 536 nm of wave length. Molar absorption coefficient for the conjugate MDA:TBA is 1.56×105 l/mol×cm.

The measurement of MDA concentration was made in duplicates, so the mean values of MDA levels were used for the calculations.

Statistical significance was determined by comparing mean values with Student's t-test for dependent samples. For normality testing, Shapiro-Wilk's test was applied. Analyzed data are shown in box plots. Statistical significance was determined at the level of $p<0.05$, with SPSS statistical software (version 18).

Results

Serum samples used in this research were taken from patients with SLE at the Institute of Rheumatology, Clinical Centre Serbia. The concentration of MDA was measured before therapy initiation (control), and after the 3-week treatment with methylprednisolone (experimental group I) and methylprednisolone+vitamin C (experimental group II).

Comparing mean levels of MDA before and after the 3-week treatment with prednysolone in patients with SLE with Student's t-test for dependent samples showed highly statistically significant decrease in concentrations (10.57±2.50 vs. 6.14±1.42; t=7.472; p<0,001).

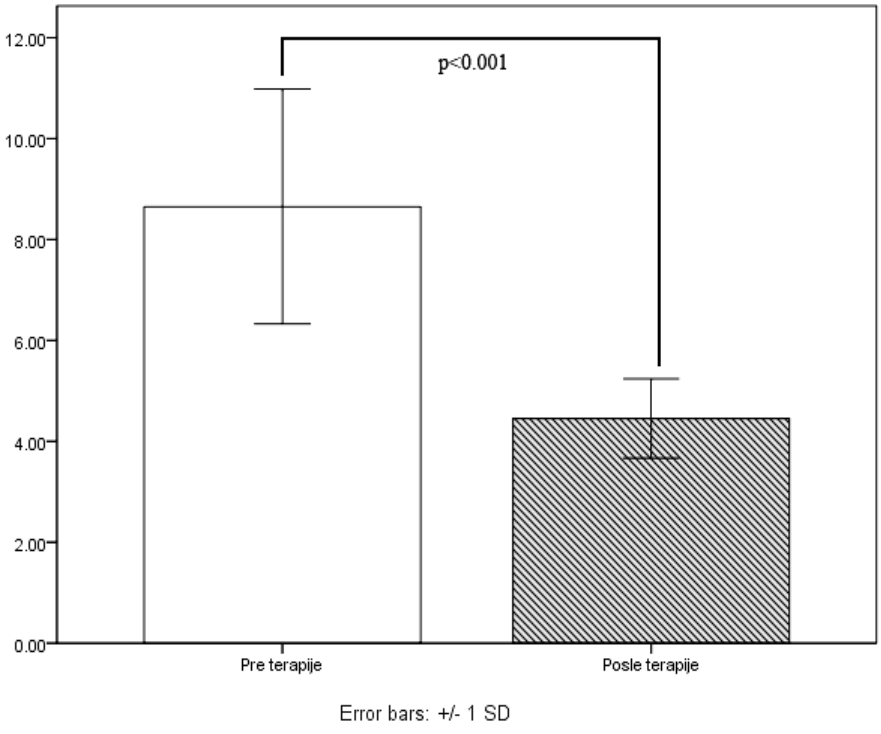

Figure 1. Mean levels of MDA before and after methylprednisolone therapy

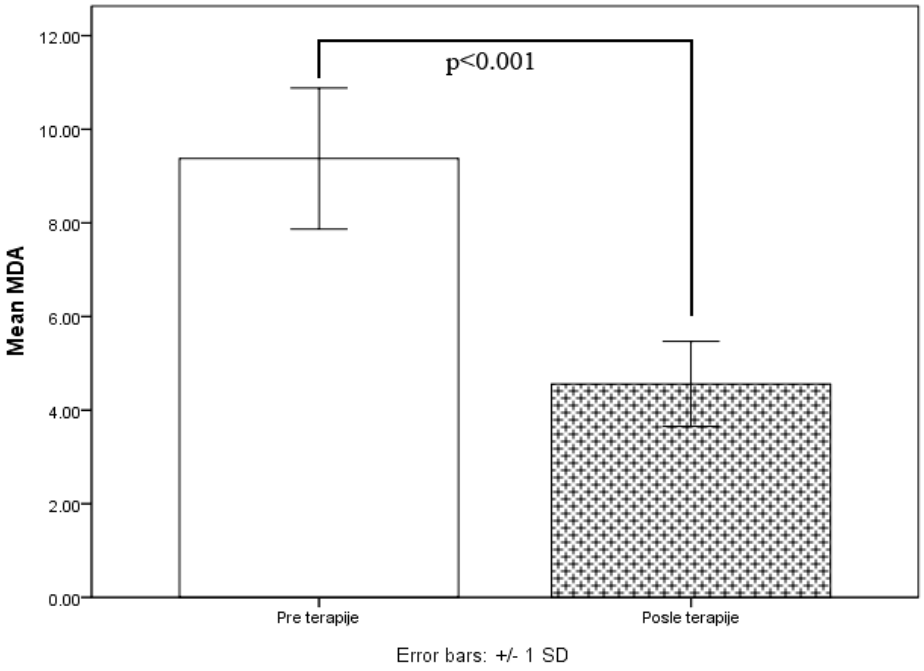

Figure 2. Mean levels of MDA, before and after therapy with methylprednisolone and vitamin C

Mean concentration of MDA before treatment with methylprednisolone was 9.06±1.93 (x±SD). After the treatment, the mean level statistically significantly decreased, and it was 4.42±0.75 (x±SD) (t=8.54; p<0.001).

After the treatment with methylprednisolone and vitamin C, a statistically significant decrease in MDA levels was observed. Initial concentration was 9.2±1.51 (x±SD) and after the treatment it lowered to 4.56±0.95 (x±SD) (t=11.793; p<0.001).

There is no statistical significance in MDA concentrations after appropriate treatment between group I (therapy with methylprednisolone) and group II (therapy with methylprednisolone and vitamin C).

Discussion

Biological oxidation of a substance that goes under catabolic changes in the organism means giving electrons away, and it is always followed with the reduction of another substance. Oxidative processes are fundamental for some basic biochemical processes in a cell, such as oxidative phosphorilation in mitochondria or microsomes, phagocytosis, lipid peroxidation of polyunsaturated fatty acids (PUFA), etc. During these reactions, small amounts of the so-called free radicals (FR) are generated. Those are atoms, molecules or ions with one or more unpaired electrons which make them extremely unstable and reactive. Beside those endogenous reactions, FR may be the result of ethanol metabolism or the absorption of ionizing, ultraviolet or heat radiation, or they may be products of nitric oxides.

The mechanism of oxidative tissue damage includes three steps: 1. generation of FR; 2. generated FR induce the formation of even more reactive oxidants; and 3. oxidant damage and inactivated macromolecules (19, 20, 21). Besides being very reactive, FR play a role in the initiation of lipid peroxidation in the cell membranes, which, later, goes on by an autocatalytic mechanism with formation of new FR, and ends with the irreversible damage of function and structure of the cell because of the PUFA accumulation (22). ROS degrade PUFA and one of the end-products is MDA.

MDA is a physiologic ketoaldehyde, a product of peroxidative degradation of unsaturated lipids, i.e. a secondary product of arachidonate metabolism. Elevated MDA concentrations are the result of tissue damage in various diseases. Plasma MDA level in a healthy adult is between 0.28 and 6.00 nmol/ml, and it may vary upon the measurement technique used (23). The level of MDA is gender-dependent, and rises with age (24) and during pregnancy (25).

There is an increase in MDA concentration in patients with SLE (26,27) in correlation with the index indicating the activity of the disease (26). High MDA level in patients with SLE has an important role in the pathogenesis of SLE, and it shows the intensity of oxidative damage. Corticosteroid application (methylprednisolone) in this research resulted in a statistically significant decrease of MDA concentration (p<0.001) in patients with SLE. It is obvious that the patients included had high MDA levels and after the treatment with corticosteroid they were significantly lower. Methylprednisolone has a suppressive effect on lipid peroxidation, but the underlying mechanism is not the direct radical scavenging or the prevention with antioxidants, but it is enrolled into suppression of lipid reactions on the

fatty acid chain and, therefore, inhibition of superoxide anion generation (28).

Clinical benefit has been registered in patients with SLE after the 3-week treatment with corticosteroids, and there was also a consecutive decrease in MDA concentration, which suggests, indirectly, the significance of FR presence in the pathogenesis of this illness.

It is interesting that the vitamin C application, as an antioxidant application, does not have a significant influence on MDA level in the patients that were already on corticosteroid therapy. Thus, some authors have shown that vitamin C supplementation leads to a better course and prognosis of SLE, and also to decrease in MDA concentration (29).

Conclusion

Our results show that oxidative products of lipid peroxidation have a significant role in the onset and chronic course of SLE. Cell damage caused by ROS generated due to intense lipid peroxidation and lowered antioxidative defenses may be stopped by corticosteroid drugs' use. Corticosteroid therapy is one of the choices for patients with inflammatory autoimmune disease. In such a way, a large number of patients are enabled to avoid the worst effects of those autoimmune diseases (renal failure, ankylosis, premature atherosclerosis). Vitamin C supplementation does not potentiate the lowering effect of methylprednisolone on MDA level in patients with SLE.

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EFEKAT LEČENJA METILPREDNIZOLONOM I VITAMINOM C NA KONCENTRACIJU MALONDIALDEHIDA KOD BOLESNIKA SA SISTEMSKIM LUPUS ERITEMATODESOM

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Naši rezultati ukazuju da oksidativni produkti lipidne peroksidacije imaju značajnu ulogu u nastanku i hroničnom toku sistemskog lupus eritematodesa. Ćelijsko oštećenje izazvano kiseoničnim radikalima nastalim zbog intenzivne lipidne peroksidacije i smanjene antioksidantne zaštite može biti zaustavljeno kortikosteroidnim lekom. Terapija kortikosteroidom je jedna od mogućnosti za bolesnike koji imaju zapaljensku autoimunu bolest. Na taj način se omogućava velikom broju bolesnika da izbegnu nastanak najtežih štetnih efekata tih autoimunih bolesti (bubrežna insuficijencija, ankiloza, prevremena ateroskleroza). Primena vitamina C ne pojačava efekat smanjenja koncentracije MDA kod bolesnika sa SLE koji se leče metilprednizolonom. *Acta Medica Medianae 2010;49(4):10-15.*

***Ključne reči**: malondialdehid, oksidativni stres, metilprednizolon, vitamin C, lupus eritematodes*