E. MURAWSKA-CIALOWICZ, L. JANUSZEWSKA, J. ZUWALA-JAGIELLO, J. MILCZARSKA, M. ZAWADZKI, M. PAPROCKA-BOROWICZ, I. WIERZBICKA-DAMSKA

MELATONIN DECREASES HOMOCYSTEINE LEVEL IN BLOOD OF RATS

1Department of Physiology and Biochemistry, University of Physical Education, Wroclaw, Poland; 2Department of Hygiene, Medical University, Wroclaw, Poland; 3Biochemistry Department, Medical University, Wroclaw, Poland; 4Biochemical Laboratory, Clinic of Heart Surgery, Wroclaw, Poland, 5Physiotherapy Department, Medical University, Wroclaw, Poland.

Elevated plasma homocysteine level promotes atherosclerosis in blood vessels due to, among others, generation of reactive oxygen species and reduction of nitric oxide bioavailability. The aim of this study was to investigate whether melatonin administration reduces plasma homocysteine level in rats consuming increased doses of methionine in the diet. The trial lasted for two months. The rats were divided into a few groups - 2 groups consisted of animals fed a standard diet, 2 groups consisted of animals fed a diet rich in methionine for one and two months, a group which had methionine removed from the diet in the second month, a group which had methionine removed from the diet and melatonin administered in the second month, a group still fed a diet rich in methionine in the second month and also given melatonin, and a group of animals on a diet rich in methionine for two months and given melatonin at the same time. Hcy, lipid peroxidation markers (MDA+4HNE) and nitric oxide metabolite (NO2-/NO3-) concentrations were determined in the plasma of all the rats. As a result of the tests it was found that plasma Hcy concentration increases in the first month of a methionine-rich diet but then decreases in the second month. MDA+4HNE changes are similar. Melatonin significantly intensifies the effects. The changes of NO2-/NO3- concentrations were noticed especially in the groups receiving melatonin. Elimination of methionine from the feed does not change the value of NO2-/NO3-. NO production increases only after administration of melatonin. On the basis of received results it might be stated that melatonin administration together with a methionine-rich diet significantly decreases Hcy concentration, the level of oxidative stress and increases NO production. It might have some practical implications, especially when the level of endogenous melatonin decreases e.g. in elderly people or people with hyperhomocysteinemia.

Key words: homocysteine, melatonin, lipid peroxidation, nitric oxide, blood, rats
INTRODUCTION

Homocysteine is non-protein highly reactive amino acid containing sulfur in its structure. It was discovered by de Vegneda in 1932 (1). It is generated due to the metabolism of methionine consumed in the diet. The process of homocysteine synthesis is called the activated methyl group pathway. Its main role is generation of methyl groups used in numerous metabolic processes e.g. synthesis of nucleic acids, lipids and polyamides. The metabolism of Hcy stands at the crossing of two pathways - remethylation, the process leading to the reconstruction of the methionine particle and transsulfuration taking place during an excessive homocysteine supply. Transsulfuration is Hcy and serine condensation (1, 2-4). The increased Hcy synthesis and its slower intracellular utilisation cause increased flux into the blood. Hence, plasma Hcy concentration is an important reflection of methionine metabolism and the rate of processes modified by folic acid, vitamins B6 and B12 as well as different enzyme activity (3). Normal plasma Hcy concentration fluctuates between 5 and 15 µmol/L. Mild hyperhomocysteinemia can be observed when values range from 31 to 100 µmol/L, severe (defined as homocystinuria) above 100 µmol/L (1, 5, 6). Homocystinuria is after phenyloketonuria the second most frequent metabolic disorder of amino acids (1).

According to the analysis a 5µmol/l increase in Hcy concentration elevates the risk of ischemic heart disease 1,6 to 1,8 -fold (7). In 1975 McCully and Wilson proposed the homocysteine theory of atherosclerosis (8) and in 1991 Genest et al. considered homocysteine an independent risk factor for atherosclerosis (7, 9). Hcy influence on the development of atherosclerosis is multidirectional. It disturbs lipid changes - favours LDL oxidation and their intake by macrophages, effects endothelial cells cytotoxically and causes their damage (10-12), increases vascular smooth muscle cell proliferation. It also disturbs the process of clot formation and fibrinolysis - inhibits C protein activation, decreases antithrombin III level, inhibits prostacyclin synthesis, facilitates platelet aggregation, increases fibre formation and atherosclerotic plaque calcification (10, 13). It destroys elasthine and increases the number of adhesive particles (10, 14). It was found that elevated homocysteine level favours formation of reactive oxygen species. They take part in the development of atherosclerosis, cause lipid peroxidation and initiate inflamation in the vascular endothelium (11, 15-18).

Melatonin is secreted by the pineal gland and was isolated in 1958. It is serotonin - 5-methoxy-N-acetyltryptamine - derivative widely spread in the flora and fauna of the world where it plays an important role in circadian rhythms (19-21). Melatonin secretion is rhythmical (19, 20, 22, 23). Peak output of melatonin takes place between midnight and 2 a.m. Light inhibits melatonin excretion. The highest melatonin level can be seen in children (3 - 5 y.o.), between 13 and 15 its level drops by approx. 80%, in people 40 - 50 y.o. a further slow reduction of melatonin level can be seen. Plasma half time is 10 minutes. Its metabolism takes place in the liver where it is conjugated with sulfates (19, 20, 23) It has recently been discovered that
it can be found in many tissues of the body, e.g. the placenta, kidneys, the respiratory tract, ventricles, the digestive system (stomach, liver, gall bladder, intestines), where its amount is much larger than in the pineal gland (24). It protects the pancreas from neoplastic changes and regulates its endocrine function. It fulfills a gastroprotective function relieving oxidative stress but also accelerates the healing process of gastric ulcers activating the COX-PG system (25, 26).

Melatonin is a strong antioxidant (27-32). It directly sweeps away free oxygen radicals OH−, ONOO−, NO−, LOO−. Melatonin eliminates them also indirectly through stimulation of SOD, CAT, GPx antioxidative enzymes and an increases of GSH concentration (29, 32). It limits the generation of free radicals formed during the Fenton's reaction by chelating metal ions of transition groups (Cu2+, Fe2+) - thanks to it melatonin limits lipid, protein and DNA peroxidation process. It limits pathological states developed on the basis of oxidative stress i.e. neurodegenerative diseases (e.g. Alzheimer's disease or tumors) (26, 33).

Cell membrane lipid peroxidation reaction is the best known destructive effect of activity of free oxygen radicals. It causes oxidation of double bonds in unsaturated fatty acids, which favours formation of gaseous hydrocarbons or aldehydes - e.g. malonyldialdehyde or 4-hydroxy-2,3 trans-nonenal.

The main aim of the study was to investigate whether exogenous melatonin decreases plasma homocysteine level after hyperhomocysteinemia evoked in rats by administration of methionine in the drinking water.

The other aim was to investigate oxidative stress level measured on the basis of lipid peroxidation markers - MDA+4HNE in animals fed melatonin and the evaluation of nitric oxide metabolites' concentration changes in the blood of tested animals.

MATERIAL AND METHODS

Chemicals

Methionine and melatonin manufactured by Sigma company (Chemical Co. St. Louis, USA) and reagent kits manufactured by Calbiochem company (La Jolla, CA, USA) and OXIS (OXIS International Inco, USA) were used in the experiment.

Animals

Studies were conducted in Wistar rats weighing 200 - 250g. The rats meant for the study were kept according to the requirements set up by the local Ethics Committee on Animal Experiments. They were kept in a temperature and humidity controlled environment on 12-h light/dark cycles.

Experimental protocol

The experiment was carried out in two stages and lasted for two months. One hundred and twenty rats divided into groups were used in the experiment. One group (n=75) consisted of animals which in the first month were fed a standard diet and given methionine in the drinking water - concentration
1,5 g/L. The aim of such a diet was to cause hyperhomocysteinemia. After one month 15 rats were randomly chosen from the group and killed. This group was marked with the DM(I) symbol. Hcy, MDA+4HNE and NO₂-/NO₃⁻ concentrations were determined in the rats' blood. During the second month the remaining animals (n=60) were divided into 4 groups each consisting of 15 animals and were marked with DM(II), D(II), D+MEL(II), DM+MEL(II) symbols, their diet was modified in the following way: Group DM(II) was fed the same standard diet together with methionine in the drinking water in concentration of 1,5 g/L in the second month. Group D(II) was only fed a standard diet without methionine in the drinking water in the second month. Group D+MEL(II) was fed a standard diet without methionine in the drinking water and was given s.c. injections of melatonin in 10 mg/kg b.w. every other day. Group DM+MEL(II) was fed the same diet as in the first month (standard diet together with methionine in the drinking water - concentration of 1,5 g/L). Additionally in the second month s.c. injections of melatonin in 10 mg/kg b.w. were given every other day to animals in the DM+MEL(II) group. The animals which, for two months, were fed a standard diet and given methionine in the drinking water in the concentration of 1,5 g/L together with melatonin administered s.c. in a 10 mg/kg b.w. dose every other day were in the separate DM+MEL(I+II) group. The animals which, for two months, were fed a standard diet and drinking water for one month were the control I - C(I), group (n=15). The animals fed a standard diet and drinking water for two months were the control II - C(II), group (n=15). When the experiment was over the animals were killed and Hcy, MDA+4HNE and NO₂-/NO₃⁻ concentrations in plasma were determined. The standard diet consisted of blended well-balanced pelleted feed for laboratory animals. It contained shelled oat, wheat, maize, soya pellets, potato protein, milk, linseed and minerals together with vitamins.

**Biochemical analysis**

Biochemical analysis was conducted with blood samples. EDTA plasma samples were used for the determination of Hcy, lipid peroxidation markers and NO₂-/NO₃⁻. After plasma isolation the samples were stored at -86°C. On the day of performing the assays the plasma samples were thawed at room temperature and their Hcy level was determined by an immunoenzymatic method with a ready-to-use reagent kit manufactured by Abbott Laboratories and with use of an AXSYM analyser. The level of MDA+4HNE was determined by a colorimetric method with use of a Lipid Peroxidation Assay reagent kit (Calbiochem). Concentration of NO₂-/NO₃⁻ was determined using the Griess reaction reagents (a ready-to-use reagent kit manufactured by OXIS).

All assays were conducted according to reagent kit manufacturers' instructions.

**Statistical analysis**

Received results were subject to statistical analyses carried out with Statistica 7.0 PL software (StatSoft, Cracow, Poland). The differences were assessed by Mann Whitney’s U test for non-normally distributes variables. Differences were examined with p ≤0.05 confidence level.

**RESULTS**

Plasma homocysteine concentration after one month of methionine administration increased significantly in comparison to the control group and was characterised with the values typical for mild hyperhomocysteinemia (Fig. I). However, Hcy concentration in the DM(II) group, where the rats were fed a diet rich in methionine for two months, significantly decreased in relation to the DM(I)
In the second month of the experiment concentration of homocysteine in the D(II) group decreased to the concentrations comparable with both control groups and values much lower than in the DM(I) group. In the second month of the experiment in the D+MEL(II) group homocysteine concentration also decreased. Values were significantly lower in comparison to all the measured groups. Homocysteine concentration in the DM+MEL(II) group was significantly lower in comparison to the DM(I) group, but remained higher in comparison to the C(I), C(II), D(II) and D+MEL(II) groups. Homocysteine concentration in the DM+MEL(I+II) group was also significantly lower in comparison to the DM(I) and DM(II) groups and higher in comparison to the C(I), C(II), D(II) and D+MEL(II) groups.

After one month of administration of methionine rich diet to rats, malonyldialdehyde and 4-hydroxyalkenals concentration raised significantly in comparison to the control group (Fig. 2). However, after two months of a diet rich in methionine concentration of lipid peroxidation markers significantly decreased in comparison to the value received for the DM(I) group. In comparison with the control groups there were no statistically significant changes. In the D(II) group it decreased significantly in comparison to almost all the groups apart from the D+MEL(II) group in which the value of MDA+4HNE was the lowest in comparison to all the groups. MDA+4HNE level in the DM+MEL(II) group
**Fig. 2.** Measurement of MDA+4HNE plasma levels in respective groups of rats (see description in Material and Methods).

\* p<0,05 in comparison to the all measured groups, **p<0,05 in comparison to the all measured groups, ***p<0,05 in comparison to the controls, DM (I), DM (II) and DM+MEL (II), DM+MEL (I+II) groups

**Fig. 3.** Measurement of nitric oxide metabolites plasma levels in respective groups of rats (see description in Material and Methods).

\*p<0,05 in comparison to the all groups, **p<0,05 in comparison to the all groups
decreased significantly in comparison to the DM(I) and DM(II) groups but without any changes in comparison to the D+MEL(II) group. In the DM+MEL(I+II) group the level of lipid peroxidation markers was significantly different in comparison to all the groups without DM(II).

Concentration of nitric oxide metabolites in the DM(I) and DM(II) groups did not differ significantly in comparison to the C(I) and C(II) groups (Fig. 3). In the D(II) group metabolites’ concentration remained at the level of values comparable with controls and did not differ in comparison to the DM(I) and DM(II) groups.

Concentration of \( \text{NO}_2^-/\text{NO}_3^- \) in the D+MEL(II) group raised significantly in comparison to all the measured groups. The values received for the DM+MEL(II) group did not differ in comparison to the control groups and also to the DM(I), DM(II) and D(II) groups. However, they were significantly lower than the values received for the D+MEL(II) and DM+MEL(I+II) groups. In the DM+MEL(I+II) group nitric oxide metabolite level was significantly higher than the values obtained for all the measured groups.

DISCUSSION

The relation between elevated homocysteine level and atherosclerosis was proved in many epidemiological studies (9, 34, 35). Currently it is known that slightly elevated homocysteine level (>16 \( \mu \)mol/L) is quite frequent in the general population and observed in about 30% of patients with cerebral, coronary and peripheral vessel diseases (8, 35-37). Higher Hcy concentration may also accompany different physiological and pathological conditions in which vitamin deficiencies are found. Pregnancy, old age, nicotinism, alcoholism, oral contraceptives etc are usually such vitamin deficiency conditions (34, 38-40).

The main mechanisms of homocysteine toxicity are auto-oxidation and generation of free oxygen radicals (39, 41-43). It is believed that sulfone groups of thiols undergo oxidation in the presence of transitional groups of metal ions and oxygen molecules. Reactive oxygen species formation is the consequence of this oxidation. Hcy as a thiol compound in the presence of transitional groups of metal ions undergoes auto-oxidation. \( \text{H}_2\text{O}_2 \) and \( \text{O}_2^- \) formation is the consequence of the process which favours lipid peroxidation and initiates inflammatory processes, endothelial damage and decreases NO bioavailability weakening vascular smooth muscle relaxation. These changes also influence antioxidative enzyme activity (44, 45).

In our studies lipid peroxidation in the blood of rats increased in the DM(I) group in comparison to the value noticed for the control groups after administration of a diet rich in methionine. However, in the second month concentration of peroxidation markers decreases to the values comparable to the concentrations noticed in both control groups. It might suggest that long-standing hyperhomocysteinemia stimulates adaptive processes, the level of oxidative
stress decreases, and thus MDA+4HNE concentrations also decrease. While analyzing the changes of oxidative stress parameters one should always take into consideration the existence of intra- and extracellular antioxidative protection mechanisms which aim is limiting oxidative changes. In our studies a month-long exposure to methionine probably stimulated adaptative mechanisms in the form of increased activity of antioxidative protection enzymes and the results of those mechanisms could be noticed in the second month of the research. Campolo et al. (46), while examining antioxidative protection after methionine administration found that it stimulates antioxidative protection and especially a production of blood reduced glutathione (GSH) through its influence on the transcription of gamma-glutamylcysteine synthase gene. That is why, paradoxically, during hyperhomocysteinemia one can notice lower MDA concentration than during normohomocysteinemia. Romerio et al. (47) also noted a relatively low increase in lipid peroxidation markers after methionine administration - only 16.7%.

Lots of authors claim that toxic effect of homocysteine on the endothelial cells is the consequence of oxidative stress (36, 37, 39, 44, 47). Numerous studies prove that Hcy decreases nitric oxide bioavailability without changes in the activity of eNOS (41, 42, 45). The mechanism of NO bioavailability limitation by homocysteine is based on the reaction between O$_2$\(^{-}\) and NO. As a result of the reaction an anion is generated with strong oxygenative and cytotoxic features - peroxynitrite (ONOO$^-\$). Due to this reaction homocysteine inhibits NO, and thus weakens vascular relaxation. Some authors think that also H$_2$O$_2$ evokes vascular contraction and additionally it disturbs NO production (48, 49). Jin et al. (50) also stated that Hcy damages function of the system y+ which is responsible for transport of L-arginine to endothelial cells. That situation additionally limits the production of NO by eNOS and increases the production of O$_2$\(^{-}\), which intensifies the destructive effects of oxidative stress. In our studies concentration of nitric oxide metabolites NO$_2^-$/NO$_3^-\$ did not increase after administration of a diet rich in methionine. So it can be thought that NO production after administration of the above-mentioned diet did not change, however, its bioavailability was inhibited due to generation of free oxygen radicals, as a consequence of a distinct increase of homocysteine concentration and lipid peroxidation markers in this group of rats as compared to the control group. Homocysteine concentration in the blood of this group of rats exceeded 4 times the values noticed in the C(I) group. In the second month of a diet rich in methionine Hcy concentration was not as high as after the first month. It seems to be quite an unexpected relation. We had expected a further increase in Hcy concentration in the second month but instead a decrease was noticed, and also a decrease in concentration of peroxidation markers without any changes in nitric oxide metabolite concentration. Mariotti et al. (51) noted a similar relation. While examining the dynamics of Hcy concentration changes in rats fed on methionine for 6 weeks, they noticed an increase in Hcy concentration in the blood up to the third week and then a significant decrease from the fifth week. The authors
suggest that a decrease of Hcy level is the result of the appearance of adaptative mechanisms connected with the changes in its metabolism regarding mostly the intensification of the transsulfuration process. Finkelstein and Martin (52) are of a similar opinion and they claim that together with a higher supply of methionine in the diet, there are changes in the activity of enzymes, especially cystathionine-β-synthase in the cells of the liver as well as the concentrations of some of the metabolites of methionine changes. These adaptative processes are supposed to lead to faster transsulfuration and faster Hcy elimination from the organism.

Cells that have protective mechanisms against oxidative damage caused by reactive oxygen species, especially $\text{H}_2\text{O}_2$, activate two independent intracellular enzymes - GPx and CAT. It was noticed that there is a clear relation between a GPx activity drop and the appearance of atherosclerosis (53-55). Buczynski et al. (56) proved that patients with vascular system diseases are characterised by the lower activity of that enzyme. Homocysteine decreases both mRNA GPx expression and the activity of this enzyme. Therefore, increased amounts of free oxygen radicals are not removed quickly enough from the environment, which causes endothelial damage. Endothelial GPx form sweeps away both hydrogen peroxide and lipid peroxides converting them into water particles and corresponding alcohols, which prevents NO oxidative inactivation (10, 39, 53, 54).

In our studies homocysteine concentration distinctly decreased in the second month of the experiment after elimination of methionine from the diet and administration of melatonin. Significantly lower homocysteine concentration in comparison with the DM(I) group was also noticed in the group of rats which were given melatonin but were still consuming methionine in the same dose as in the first month. A practical conclusion results from this fact. Melatonin administered together with a diet rich in methionine may decrease homocysteine level and counteract atheromatous changes. Studies by Okatani et al. (48, 49) prove that melatonin inhibits homocysteine auto-oxidation by sweeping away $\text{O}_2^-$ radical. Melatonin is known as a powerful scavenger of, among others, superoxide radical anion, hydrogen peroxide and peroxynitrite (22, 30, 31, 32, 57). The other important mechanism of melatonin action which weakens homocysteine effect is glutathione peroxidase activity stimulation, which increases NO bioavailability and facilitates vascular smooth muscles relaxation (32, 57, 58). In our studies in the group of rats which had methionine eliminated and were given melatonin together with a standard diet, lipid peroxidation decreased in comparison to the control group and to the group which was fed a methionine-rich diet. Similar effects were noticed in the DM+MEL(II) group, which in the second month of the experiment received melatonin apart from methionine. $\text{NO}_2^-/\text{NO}_3^-$ in the D+MEL(II) group significantly increased in comparison to the control groups and the group of animals fed on methionine for one and two months. It proves that melatonin is a very efficient antioxidant also when a diet is rich in methionine. Results similar to ours were achieved by Baydas et al. (59). They noticed a decrease in homocysteine level and a decrease (statistically significant in
comparison to the control) in concentration of lipid peroxidation markers after administration of melatonin to rats.

It should be remembered that melatonin is not only a strong antioxidant secreted by the pineal gland. Its high concentration has also been found in the cells of other tissues, kidneys, the respiratory tract, the pancreas, the liver, the gall bladder, the stomach, the duodenum (24-26, 60). In the digestive system it fulfills its gastroprotective function. It protects from damage caused by oxidative stress, accelerates the healing process of gastric ulcers activating the COX-PG system (25), and regulates the endocrine function of the pancreas (24, 60). It also seems important that its concentration in the blood increases significantly during consumption of food in general and especially of food that contains tryptophan (24). Thus, it might be assumed that to some extent it might also affect Hcy metabolism and limit its synthesis. However, it is only a conjecture and requires further research.

Our results as well as the results of the quoted authors confirm speculations that melatonin supplementation decreases plasma homocysteine level in a distinct way, reduces the level of oxidative stress. The knowledge of this biological effect can be used to treat atherosclerosis and cardiovascular diseases which are caused by atherosclerosis.

Conflicts of interest statement: None declared.

REFERENCES


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Author’s address: Eugenia Murawska-Cialowicz, Physiology and Biochemistry Department, University of Physical Education in Wroclaw, I.J. Paderewskiego 35 Street, 51-612 Wroclaw, Poland, phone: 0048 71 347 33 59; fax: 0048 71 347 30 36; e-mail: eugenia@awf.wroc.pl