

EXPERIMENTAL STUDY

The effects of different doses of melatonin on lipid peroxidation in diet-induced hypercholesterolemic rats

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Abstract: This study aims to see in an animal experiment how differently the low and high doses of melatonin affect the antioxidant status and peroxidation of lipids. Forty-two male Wistar-Albino rats weighing about 200 gr (180–220) aged 6–7 months were used. Of these rats, 12 were fed with normal rat chow for 12 weeks. The latter ones were divided into two groups, each containing 6 rats. Group 1 (control group) received daily intraperitoneal injections of NaCl (0.9%; w/v). Group 2 was injected ethanol daily (4%; v/v; i.p.) to see the effects of ethanol in which we dissolved melatonin. Thirty rats were fed with a diet enriched with cholesterol (2%; w/w), cholic acid (0.5%; w/w) and propylthiouracil (0.5%; w/w) for 12 weeks. These rats were divided into three groups each containing 10 rats. The low-dose group received melatonin 1 mg/kg/d; i.p. (group 3), the high-dose group received melatonin in a dose of 10 mg/kg/d; i.p. (group 4), and only the cholesterol group did not get any vehicle (group 5). Total cholesterol (TC), LDL cholesterol (LDL-C), total antioxidant capacity (TAC), oxidized LDL (oLDL) and TBARS levels were measured in all groups. The produced high-cholesterol diet increased LDL cholesterol. Melatonin decreased the extent of this plasma lipoprotein increase and also prevented the oxidation of it. This effect was clearer when the dose was higher. Antioxidant status seems to be also dose-dependent (Tab. 2, Ref. 33). Full Text in PDF www.elis.sk.

Key words: melatonin, oxidized LDL, lipid peroxidation, hypercholesterolemia, TBARS.

Coronary artery disease (CAD) is one of the leading causes of mortality and morbidity all over the world (1, 2). Traditional risk factors of CAD are age, sex, hypercholesterolemia, arterial hypertension, diabetes and smoking. One of the strongest risk factors is the high concentration of LDL-C (3). LDL particles are oxidized by free radicals in the intimal space of arteries. The oxidative modification of LDL increases its uptake by macrophages via scavenger receptors. This process ends with the formation of foam cells and atherosclerosis (4).

Melatonin secreted by the pineal gland is a powerful scavenger of free oxygen radicals, especially OH radicals and probably peroxy radicals (5). There is also evidence that melatonin is also an indirect antioxidant as it promotes activities of a variety of antioxidative enzymes (6, 7–12). Exogenous melatonin has been shown to reduce the lipids. Plasma levels of LDL-C and TC were reduced by melatonin in hypercholesterolemic rats (13–16).

Oxidized low-density lipoprotein (oLDL) is an important factor in the initiation and progression of atherosclerosis (2). It leads the endothelial dysfunction and plaque destabilization through multiple mechanisms. LDL particles accumulate in the intimal space of arteries and are oxidized by free oxygen radicals. As

a result of lipid peroxidation, several non-radical products are formed, including malondialdehyde (MDA) and other reactive aldehydes. Melatonin, its precursors, as well as its breakdown products have been shown to prevent the oxidation of lipids (17). Because of all these hypolipidemic and antioxidative effects, this pineal indole has been used in many experimental studies as a vehicle in different ways and in different doses. It is suggested to be a physiological antioxidant by experimental data (12–16). In addition, a correlation between melatonin levels and blood antioxidant status has been suggested. Therefore, we aimed to add some new evidence about the latter by using melatonin in both low and high doses.

Materials and methods

Design of the experiment

Interventions concerning animals were performed according to the Guide for Care and Use of the Laboratory Animals. Forty-two male Wistar-Albino rats weighing about 200 g (180–220) aged 6–7 months were housed four or six per cage in a room maintained at 23–25 °C with 12-hour light-dark cycles; the lights were off daily from 18:00 through to 06:00 for 12 weeks. Of the latter rats, 12 were fed with normal rat chow and divided into two groups of 6 rats each. Group 1 (control group) received daily intraperitoneal injections of NaCl (0.9 %; w/v). Group 2 was injected ethanol daily (4 %; v/v; i.p.) (group 2) to see the effects of ethanol in which we dissolved melatonin. Thirty rats were fed with a diet enriched with cholesterol (2 %; w/w), cholic acid (0.5 %; w/w) and propylthio-

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Tab. 1. Results of serum oxidized LDL, total and LDL cholesterol, total antioxidant capacity and TBARS levels of the groups (mean±SD).

Variables	Group 1 (n=6)	Group 2 (n=6)	Group 3 (n=10)	Group 4 (n=10)	Group 5 (n=10)
TC (mg/dL)	62.63±13.83	59.71±7.42	168.31±26.6	135.79±10.83	201.8±24.43
LDL-C (mg/dL)	15.71±12.54	6.93±0.98	108.89±24.12	69.44±12.05	121.61±24.97
TAC (U/L)	252.16±11.37	253.7±23.34	346.2±33.45	375.11±21.64	231.7±29.88
oLDL (U/L)	11.2±0.67	17.68±2.33	19.46±2.03	16.61±3.3	20.51±3.82
TBARS (μmol/mL)	2.93±0.19	4.53±0.17	4.85±0.18	3.69±0.27	7.61±0.51

uracil (0.5 %; w/w). These rats were divided into three groups of 10 rats each. The low-dose group received melatonin in a dose of 1 mg/kg/d; i.p. (group 3), the high-dose group received melatonin in a dose of 10 mg/kg/d; i.p. (group 4), while only the cholesterol group did not get any vehicle (group 5). Melatonin, cholesterol and cholic acid were purchased from Sigma (St. Louis, MO, USA) and thiouracil from Dr. F. Frik. Melatonin was dissolved in absolute ethanol (4 %; v/v in 0.9 % NaCl w/v), and this solution was prepared freshly three times a week. The animals were kept in wire-bottomed stainless steel cages and were given diet and water *ad libitum*. The experimental procedures used in this study met the guidelines of the Animal Care and Use Committee of the University of Istanbul.

Biochemical analysis

Twelve weeks from the start of experiment, the rats were fasted overnight and then anesthetized with sodium pentobarbital. The blood was collected in tubes by cardiac puncture. All serum samples were frozen at –80 °C in aliquots until assayed.

Measurement of lipid parameters

Total cholesterol (TC) levels were indicated by enzymatic method (Diasis). LDL cholesterol was calculated by Friedewald Formula.

Lipid peroxidation assay

Serum oxidized LDL (oLDL) levels were measured with competitive ELISA method, which is based on monoclonal antibody mAb-4E6 by using kits from Mercodia. The degree of lipid peroxidation was estimated in terms of thiobarbituric acid-reactive substance (TBARS) value determined by malondyaldehyde (MDA) formation. Briefly, one milliliter of acid solution containing 15 % (w/v) trichloroacetic acid (TCA), 0.375 % (w/v) thiobarbituric acid (TBA) and 0.25 N hydrochloric acid (HCl) was added to the samples and the tubes were closed with taps. After incubation at 95 °C for 15 minutes, they were cooled. After centrifugation at 4,000 G for 5 min, the absorbance of the supernatant was measured at 532 nm.

Assay of antioxidant capacity

The determination of total antioxidative capacity is performed by the reaction of antioxidants in samples with a defined amount of exogenously provided hydrogen peroxide. After the elimination of a certain amount of the provided hydrogen peroxide, the residual hydrogen peroxide is determined colorimetrically by enzymatic reaction which involves the conversion of TMB into a colored product. After stopping the reaction, the samples were measured at 450 nm (ImmunDiagnostik).

Tab. 2. Correlations of serum oxidized LDL, total and LDL cholesterol, total antioxidant capacity and TBARS levels of the groups (mean±SD).

	TC	LDL-C	TAC	oLDL	TBARS
TC		r=0.977 p<0.001	r=-0.100 p>0.05	r=0.686 p<0.01	r=0.771 p<0.01
LDL-C			r=-0.080 p>0.05	r=0.675 p<0.01	r=0.752 p<0.01
TAC				R=-0.157 p>0.05	r=-0.424 p<0.01
oLDL					r=0.821 p<0.01
TBARS					

Statistical analysis

Conventional methods were used for calculating the means and standard deviation (SD). All results were expressed as mean ± SD. Statistical analysis was performed using Kruskal–Wallis test to see if there is a significant difference between the groups. Then the groups were mutually compared with the Mann-Whitney U test. For correlation analysis, Spearman's correlations were used. Values of p equal to or less than 0.05 were considered significant.

Results

There was not any significant difference between the levels of serum TC, TAC of groups 1 and 2 but the LDL-C levels were significantly lower; TBARS and oLDL levels were significantly higher in group 2 when compared with those of group 1.

In groups 3 and 4, total serum cholesterol, oxidized LDL, and TBARS levels were significantly lower; total antioxidant capacity levels were significantly higher than those in group 5. In group 4, serum TC, LDL-C, TBARS, and oLDL levels were significantly lower; TAC levels were significantly higher than those in group 3. Both of groups 3 and 4 had significantly higher TAC levels than groups 1 and 2 (Tab. 1).

According to Spearman's correlation test, there was a positive correlation between serum oLDL and LDL-C ($r = 0.675$; $p < 0.01$), oLDL and TBARS ($r = 0.821$; $p < 0.01$), as well as between LDL-C and TBARS ($r = 0.752$; $p < 0.01$) levels. A positive correlation was detected between serum TC and TBARS ($r = 0.771$; $p < 0.01$), as well as between TC and oLDL ($r = 0.686$; $p < 0.01$) levels. Besides these, we found a negative correlation between serum TAC and TBARS levels ($r = -0.424$; $p < 0.01$) (Tab. 2).

Discussion

Alongside high concentration of low-density lipoprotein (LDL), hypercholesterolemia has been implicated as a risk factor

in the development of coronary artery disease (CAD). Since the oxidative modification of LDL leads to the whole process, there has been interest to investigate the relationship between oxidative stress and CAD. Melatonin is a highly potent endogenous radical scavenger protecting the biomolecules against oxidative modification (15). Melatonin administration has been shown to have no significant effect on plasma lipids and lipoproteins in rats on normal diet but diminishes the effects of high-cholesterol diet on lipid parameters (14, 17). The protective action of melatonin was manifested only following the induction of cholesterolemia in rats (16). Therefore, as the antioxidant system is a protector in situations like hypercholesterolemia, which can induce oxidative stress, melatonin seems to be an agent playing its metabolic role harder on the lipid side. Melatonin has been studied in different diseases in which the free radicals are suspicious to be triggering the pathogenesis. Especially the cardiovascular disease is one of the most studied fields about the melatonin's beneficial effects. Nevertheless, different doses have been used in different ways (20).

We used the clinically most relevant dietary model to monitor the lipid peroxidation induced by hypercholesterolemia and found that melatonin prevented peroxidation by its antioxidative properties. The purpose of this study was to investigate two different doses of melatonin. Şener et al. have found an intraperitoneal dose of 10 mg/kg of melatonin, to be protective against oxidative stress in a sepsis model (19). The same dose of melatonin was also used by Nam et al. in neuronal damage and found that melatonin depressed the lipid peroxidation nearly to the control levels (21). While these two studies were designed to be short term, Balkan et al were giving melatonin (10 mg/L in drinking water) to rats for 4 months. They found that the melatonin treatment had an ameliorating effect on the disturbances in prooxidant-antioxidant balance in the liver of mice following cholesterol feeding (22). Administration of melatonin (10 mg/kg/day) for 4 weeks in cadmium intoxicated rats diminished the levels of oxidative stress markers, lipid peroxidation, and protein carbonyls in brain while elevating the levels of nonenzymatic and enzymatic antioxidants (23). Considering the high dose of melatonin, these results were similar to ours. The present study demonstrates that high doses of melatonin lower the total and LDL cholesterol and oxidation parameters much more than low doses do. Besides this, the rats receiving a high dose have much more antioxidant capacity than those in the low-dose group. Group 2 received ethanol, and the results showed the well-known hypolipidemic effect of alcohol, especially on LDL-C. However, groups 3 and 4 received cholesterol-rich diet that enhanced the lipids as seen in group 5. The ability of melatonin to lower LDL-C even in such a diet is inferred from our results. Lipid peroxidation is a basically degenerative reaction and its products such as TBARS are toxic to cells (24–26). As mentioned above, melatonin has been shown to be highly effective in protecting against oxidative damage caused by several factors such as aging, ischemia-reperfusion, toxins and hypercholesterolemia (17, 27–30). The present data also indicate the oxidant activity of ethanol on lipids. In the present investigation, in groups 3 and 4, melatonin was found to reduce both the oxidized LDL

and TBARS, which are the products of lipid oxidation caused by both hypercholesterolemia and ethanol.

Conclusions

As seen, many different studies use melatonin as an antioxidant agent, however in different doses. Its hypolipidemic and antioxidant effects are very clear and the results suggest the possibility that melatonin could be clinically applied. Our results also support the clinical use of melatonin to treat conditions in which lipid peroxidation is present. Since these findings may have clinical importance, additional studies in this field should be of high priority.

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