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The Effect of Levonorgestrel and Melatonin Treatments on Plasma Oxidant-Antioxidant System, and Lipid/Lipoprotein Levels in Female Rats

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Abstract: This study was carried out in order to determine the effects of levonorgestrel (LNG) and progestin derivatives, alone and in combination with melatonin (MEL), on the plasma oxidant-antioxidant system, and also lipid and lipoprotein levels, in the rat model.

Female rats were divided into 4 groups according to subcutaneous treatment with LNG (5 mg/kg/day), MEL (25 mg/kg/day) and a LNG-MEL combination (5 mg LNG/25 mg MEL/kg/day) for 5 consecutive days, and a control group. Following the treatment period, malondialdehyde (MDA), conjugated dienes (CD), thiol (SH) and glutathione peroxidase (GPx) values, as the components of the oxidant-antioxidant system, and also triglyceride (TG) and cholesterol (TC, HDL-C, LDL-C) levels were measured in plasma obtained from the rats. Statistical comparisons were made using ANOVA and post-ANOVA tests.

There were no significant differences in respect of any of the measured parameters between the controls and the MEL group. Although there were no significant differences in TG and HDL-C, MDA and CD levels were found to be higher, but SH and GPx values lower in the LNG group than in the control and MEL groups. On the other hand, the LNG treatment along with MEL resulted in significant decreases in MDA, CD, TC, LDL-C levels and elevations in SH and GPx in respect of the LNG group, and these values measured in the LNG-MEL group were not different from those of the control and MEL groups.

In conclusion, the use of MEL together with synthetic sex steroids, which may lead to oxidative stress and induce the risk of cardiovascular disease (CVD), may play an important role in reducing the risk of CVD, through the protection of the antioxidant system.

Key Words: Levonorgestrel, melatonin, the risk of cardiovascular disease, oxidant-antioxidant system, lipid-lipoprotein profiles.

Introduction

In addition to their central actions in the hypothalamo-pituitary axis, sex steroids have direct effects on peripheral metabolic tissues. Of these, the liver is most affected by sex steroids (1). In our previous reports, we suggested that contraceptive steroids may exert their metabolic effects by changing hepatic enzyme levels related to the synthesis and/or turnover of lipids and lipoproteins (2, 3).

Several epidemiologic studies have demonstrated that alterations of plasma lipid and lipoprotein pattern brought about by contraceptive steroids may increase the risk of cardiovascular disease (CVD) (4-6). However, lipid peroxidation induced by oxygen free radicals are also thought to be involved in CVD (7, 8).

There are conflicting reports that synthetic sex steroids may increase the risk of CVD by leading to oxidative damage (9, 10), or decrease it by protecting the antioxidant system (11).

On the other hand, melatonin (MEL), a lipophilic indole amine hormone secreted by the pineal gland, has been suggested as a potent antioxidant that may protect against development of atherosclerosis, cancer and aging (12, 13).

The aim of the present study was to examine the effects of levonorgestrel (LNG, a progestin derivative), alone and combination with MEL on the plasma oxidant-antioxidant system and also lipid and lipoprotein levels in the rat model. Therefore, malondialdehyde (MDA) and conjugated dienes (CD), the products of lipid

peroxidation, and thiols (SH) and glutathione peroxidase (GPx) activity were considered indicators of the antioxidant system, and also lipid and lipoprotein levels, well known as CVD risk factors, were measured in the plasma of female rats treated with LNG and/or MEL.

Material and Methods

Material: LNG (N-2260), MEL (M-5250) and propylene glycol (PEG, P-1009) were purchased from Sigma Chemical Co., Germany. All other chemicals used were of the highest purity commercially available.

Animals: Mature, 90-120-day-old female Swiss albino rats weighing 180-230 grams were used for all groups. The rats were housed in groups of 5 in wire-bottomed, stainless-steel cages in an animal room kept at normal temperature and humidity, with a 12-hour light-dark cycle. They were fed with standard Purina rat chow and tap water ad libitum.

To ensure adequate adaptation, the animals were observed in this environment for 14 days prior to the experiments.

Experimental design: A total of 40 female rats were randomly divided into 4 groups, 10 rats in each, according to treatment with LNG, MEL, and a LNG-MEL combination, and a control group.

LNG and MEL were dissolved in absolute ethanol and these stock aliquots (0.1 ml), stored at -70°C, were diluted with PEG in suitable concentrations before the treatments.

The doses of LNG,MEL and LNG-MEL combination in 1.0 ml of PEG per kilogram of body weight, administered subcutaneously to the rats for 5 consecutive days, are presented in Table 1.

Following the treatment period, blood was taken in heparinized tubes from the abdominal aorta of the rats, which had fasted overnight with free access to water, under anaesthesia with diethyl ether. Plasma was obtained from the blood by centrifugation at 1500 g at 4°C for 20 min. The aliquots of plasma were stored at –70°C until analysis was performed. Only plasma SH measurements were carried out on the same day.

Analytical procedures: Plasma SH levels were estimated with the method of Koster et al. (14), using the thiol-disulphide interchange reaction between DTNB and thiols. Plasma MDA levels were assayed using a spectrophotometric method (15) which was partly modified in our laboratory, as previously described in the literature (16). CD levels were measured at 233 nm in plasma lipid extracts solubilized in n-heptane (17). Plasma MDA, CD and SH levels were expressed as micromoles per liter (µmol/L).

GPx activity in the plasma was determined by the coupled assay described by Paglia and Valentine (18), using H_2O_2 as substrate. The assay kinetics were calculated with activity expressed as micromoles of NADPH of 6200 $m^{-1}cm^{-1}$ at 340 nm. oxidized per min per liter of plasma (U/L).

Plasma triglyceride (TG) levels were measured enzymatically, on an automated analyzer. Total cholesterol (TC) measurements were made by the modified Leffler colorimetric method (19). HDL-cholesterol (HDL-C) was determined using a sodium tungstate-magnesium precipitation procedure (20) prior to TC measurement (19). LDL-cholesterol (LDL- C) was calculated using the formula of Friedewald et al (21). Plasma TG and cholesterol levels were expressed as millimoles per liter (mmol/L).

Statistical analysis: The significance of the data obtained from the study groups was evaluated using the analysis of variance (ANOVA) and the differences between the means were then analyzed using a post-ANOVA (Scheffe's procedure) test (22). The level of significance was taken as $p < 0.05$.

Results

The plasma MDA, CD and SH levels and GPx activities, and the plasma lipid and lipoprotein levels of the study groups are presented in Table 2 and Table 3, respectively.

There were no significant differences in respect of the measured parameters between the controls and MEL group, as seen in Table 2. Plasma MDA and CD levels were found to be higher, but SH levels and GPx activities were lower in the LNG group than in the control and MEL groups. Although the CD levels were higher and GPx activities were lower than in the MEL group, MDA, CD and SH levels, but excluding GPx activity, were not found

Table 1. Experimental Design of Study Groups.

Groups	n	Dose treated (mg/kg rat weight/day)
Control	10	1.0 ml PEG
MEL	10	25.0 mg MEL
LNG	10	5.0 mg LNG
LNG-MEL	10	5.0 mg LNG/ 25.0 mg MEL

n: The number of rats in each group.

Table 2. MDA, CD, SH Levels and GPx Activities in Plasma of Study Groups.

Groups	MDA (μmol/L)	CD (μmol/L)	SH (μmol/L)	GPx (U/L)
Control	8.15 ±2.09	150.08 ±15.71	491.62 ±76.44	498.71 ±81.22
MEL	7.66 ±1.36	126.03 ±17.99	513.24±89.58	520.90 ±56.61
LNG	11.24± 2.50$^{*,\bullet}$	209.36±45.43$^{*,\bullet}$	347.65±62.67$^{*,\bullet}$	280.70±60.74$^{*,\bullet}$
LNG-MEL	8.98 ±1.38^{a}	166.42±18.43$^{\#,a}$	429.26 ±72.35	383.66±62.56$^{*,\#\#,a}$

The values are mean ± SD for 10 rats in each group
Statistical comparisons were made
- Between parameters of control and study groups ($^{*}p < 0.01$)
- Between study groups : MEL vs LNG ($^{\bullet}p<0.01$)
MEL vs LNG-MEL ($^{\#}p<0.05$; $^{\#\#}p<0.01$)
LNG vs LNG-MEL ($^{a}p<0.05$)

Table 3. Plasma Lipid and Lipoprotein Levels of Study Groups.

Groups	TG (mmol/L)	TC (mmol/L)	LDL-C (mmol/L)	HDL-C (mmol/L)
Control	0.59±0.12	1.29±0.15	0.40±0.06	0.62±0.11
MEL	0.62±0.09	1.25±0.13	0.32±0.06	0.65±0.10
LNG	0.64±0.13	1.49±0.14$^{*,\bullet}$	0.58±0.07$^{**,\bullet}$	0.62±0.11
LNG-MEL	0.63±0.13	1.28±0.14$^{\#}$	0.38±0.08$^{\#\#}$	0.61±0.09

The values are mean ± SD for 10 rats in each group
Statistical comparisons were made
- Between parameters of control and study groups ($^{*}p < 0.05$; $^{**}p<0.01$)
- Between study groups: MEL vs LNG ($^{\bullet}p<0.01$)
MEL vs LNG-MEL ($p>0.05$)
LNG vs LNG-MEL ($^{\#}p<0.05$; $^{\#\#}p<0.01$)

to be statistically different from the values for the controls, in the LNG-MEL group. However, decreases in the MDA and CD levels and an increase in GPx activity was observed in the LNG-MEL group when compared with the LNG group (Table 2).

Although there were no significant differences in respect of the measured TG and HDL-C levels in the study groups, there were significant increases in the TC and LDL-C levels in the plasma of the LNG group when compared with both the control and MEL groups. TC and LDL-C levels were found to be lower in the LNG-MEL group than in the LNG group; and these parameters were not significantly different from the values of the control and MEL groups.

Discussion

It is well known that the risk of CVD is related, among many factors, to elevated lipid peroxide levels (7, 8). According to the free radical theory of CVD, the equilibrium between the synthesis of prostacyclin and thromboxane, preventing and inducing platelet aggregation, respectively, is dependent on the degree of lipid peroxidation and the potential of the antioxidant system in the organism (7). Lipid peroxidation induced by oxygen free radicals may contribute to the formation of an initial lesion in the arterial wall, which may predispose development of atherosclerosis by enhancing leucocyte chemotaxis and activation and platelet aggregation (7, 8).

Several studies investigating the effects of synthetic sex steroids on lipid peroxidation have suggested different explanations. Although the antioxidant potential of the estrogens, including phenolic hydroxyl groups, has been reported (23), the suppression of the antioxidant system including enzymes such as GPx (10), and the elevation in the products of lipid peroxidation such as MDA, CD, and enhanced platelet aggregation related to the oxidative damage have also been suggested in the use of contraceptive steroids (10, 11, 24).

Most combined contraceptive steroids currently used contain ethinyl estradiol, so that any differences in their metabolic effects depend on the dose and type of progestin component (4). Since LNG, of the 19-nortestosterone derivatives, has the most androgenic, antiestrogenic and progestational activities (6), it may have more effect on metabolic interactions.

Elevated plasma MDA and CD along with reduced SH and GPx values in the present study show that lipid peroxidation is enhanced and contrary to this, the antioxidant system is weakened with LNG treatment.

The existence of positive correlations between lipoperoxides/MDA and TC and also TG in patients with atherosclerotic lesions suggests the possibility of lipid peroxide interference with other metabolic pathways (25). Of the plasma lipids and lipoproteins, LDLs are the most susceptible to peroxidation. Furthermore, the higher plasma cholesterol levels may lead to a greater increase in peroxide formation in LDLs (7). Oxidized LDL may promote the development of plaque through a number of potentially atherogenic properties. It is a chemoattractant for leucocytes and stimulates cytokine production by macrophages. Oxidized LDL has recently been considered to play an important role in atherogenesis (26).

There have been conflicting reports that LNG administered to rats increases plasma TC and LDL-C (27); decreases (27); or does not change (28) TG levels; and lowers (29) or does not affect (28) plasma HDL-C levels. La Rosa (30) suggested that changes in TG levels may depend on the estrogen component but LDL-C levels may be related to the progestin component of combinations. In addition, it has been observed that LNG treatment alters the composition of LDL in monkeys and this modified LDL is highly reactive with arterial proteoglycans. Therefore, it has been suggested that LNG has potentially atherogenic effects on LDL (31).

Although there were no changes in TG and HDL-C levels, increases in TC and LDL-C with LNG treatment were observed in the present study. When taking into consideration all the findings related to LNG treatment, it may be assumed that LNG may suppress the antioxidant system, but induce lipid peroxidation and, in this way, may affect lipid/lipoprotein metabolism. In accordance with our study, Durand et al. (24) proposed that the primary action of contraceptive steroids is the induction of oxidative stress that generates oxygenated radical species leading directly to modification of cell membranes and lipoproteins.

However, it has been suggested that preventing the oxidation of LDL in the presence of antioxidants may reduce atherogenesis (26, 32). In vitro and in vivo experiments have indicated that MEL has antioxidant potential, in addition to its known effects on endocrine function and circadian rhythm. Furthermore, MEL has been found to be the most potent scavenger for hydroxyl radicals among the antioxidants (12).

It has been reported that MEL administration to diabetic rats induced by streptozotocin decreases plasma TG, cholesterol and MDA levels, and returns erythrocyte GSH content to normal levels (33); similarly, MEL treatment reduces MDA levels in the plasma of old rats (34). In addition, there are many reports indicating that MEL has a preventive effect on LDL-oxidation (35, 36). It has also been suggested that MEL may protect the organism from the harmful effects of free radicals by stimulating GPx activitiy, whereas a decrease in GPx may lead to an increase in the degree of lipid peroxidation (37, 38).

In the present study, it was also shown that the addition of MEL to LNG treatment was sufficient to negate the alterations observed in the oxidant-antioxidant system and lipid/lipoprotein levels depending on LNG, that is, the decreases in plasma MDA, CD, TC and LDL-C levels and the increases in SH and GPx values were denoted using a LNG-MEL combination.

In conclusion, the use of MEL together with synthetic sex steroids, which may lead to oxidative stress and induce CVD risk, may play an important role in reducing the risk of CVD, through protection of the antioxidant system.

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