The effect of ethinyl estradiol/levonorgestrel (EE/LNG) combination on plasma lipoprotein metabolism in female rats (II)

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In order to determine dose-and-time dependent effects of EE/LNG on plasma lipoprotein metabolism, rats divided into 2 groups for short and long-term investigations were subdivided into 3 subgroups: Low-dose (LD) and high dose (HD) of EE/LNG and control. At the end of the experiments, triglyceride (TG), LDL-cholesterol (chol), HDL-chol, apolipoprotein (Apo) A1 and B levels in plasma and hepatic lipase (HL) activity in the liver of rats were determined.

When compared to controls, in all groups LDL-chol were increased, and except long-term LD group, plasma TG were also increased; whereas HDL-chol were decreased in HD groups. Apo A\ levels were significantly higher in short term HD EE/LNG group, while Apo A\ was increased by LD application, but it decreased by HD combination in long period. There was no significant change in Apo B during the experiment. An increase in HL activiy was observed in long-term HD group, but decreased in other groups. When the groups were compared according to dose and duration, EE/LNG effected TG and HDL-chol depending on the dose, while changed HL, LDL-chol and Apo A^ by dose and duration.

It was concluded that the effect of EE/LNG on plasma lipoprotein metabolism may be related to the changes observed in HL activity. [Turk J Med Res 1993, 11(6): 266-272]

Key Words: Lipase, Lipoproteins, Rats

It has been known that hormonal components of contraceptive steroids, estrogens and progestins, have some effect related to alterations in lipid and lipoprotein metabolism. Exogenous estrogens may increase plasma total cholesterol (T.chol), trigliserid (TG), phospholipid (PL), apolipoprotein (Apo) Ai and B levels (1,2). They also increase plasma HDL concentrations, mainly by increasing the HDL-2 subfraction (3); but decreases LDL-chol levels (4). On the other hand, synthetic progestins derived from 19-nortestosterone, depending on their antiestrogenic and androgenic activities (5), decrease plasma HDL2 and HDL levels (6), but increase LDL levels (7). In addition, these steroids that decrease plasma VLDL and TG concentrations (8) also reduce plasma Apo Ai and potantially increase Apo B concentrations (9).

Most of the estrogen effects on lipoproteins are opposite to those of progestins so that the net response to combinations is determined by the doses and potency of the particular steroids employed. Therefore, in recent years, the quantities of both estrogen and progestin have gradually been reduced (2,10). However, even very low-dose combinations were used, some alterations in lipoprotein metabolism which were in minimal levels have been observed (11,12,13). It is apparent that these alterations occured in lipoproteins and its subtractions and in apolipoprotein concentrations in a way suggesting that more subtle changes in lipoprotein composition could have been induced (6,14). As it is suspected that biochemical changes in circulating lipoproteins can alter hepatic lipoprotein synthesis or cholesterol delivery to peripheral cells or, conversely, the reverse cholesterol transport to the liver; it is felt that more detailed metabolic studies at cellular enzyme level are needed.

The purpose of the second part of this study is to investigate in the rat model the changes in plasma lipoproteins in association with contraceptive steroid use and to determine whether these changes may be related to the induction or suppression of the hepatic [illegible] activities by E/P combinations depending on the dose and treatment-duration. For this reason, TG, HDL-chol, LDL-chol, Apo Ai and B levels in plasma samples and HL activity in livers of

Received: Oct. 20,1992 **Accepted:** Sept. 23, 1993

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female rats treated with low and high doses of EE/LNG combination for short (15 days)-and long (120 days)-terms were measured.

MATERIALS AND METHODS

Material and animals used in this second part and eperimental design were the same as in the first part of the present study (15). Briefly; rats divided into 2 groups for short-and long-term investigations were then subdivided into 3 subgroups according to orally given low and high doses of EE/LNG combination. The choice of dose was performed as described earlier (16): 4 times for low-dose (LD) and 200 times for high-dose (HD) anticipated clinical dose on a milligram per kilogram of body weight. According to this, the combination containing 0.03 mg EE and 0.125 mg LNG in each pill, at low doses (2.4 ug EE/10 ug LNG) and high doses (120 ug EE/500 ug LNG), were administrated to rats by adding to their water, on a microgram per kilogram of rat weight, daily (15). At the end of the treatment periods, plasma TG, HDL-chol, LDL-chol, Apo Ai and B levels and HL activity were determined.

Analytical Methods: Plasma TG levels were meased enzymatically, on an automated analyzer. HDL-chol determination was made in the supernatant obtained from removing VLDL and LDL by using dextran sulphate (50.000)-Mg^{+2} precpitation method (17,18). LDL-chol levels were calculated from Friedewald Formula (19). [T. chol levels used in the formula were given in the first part of the present study (15)]. Plasma Apo A1 and B levels were measured by immunoturbidimetric assay (20) using kits from Orion Diagnostica.

Hepatic **Lipase Analysis:** Preparation of rat liver homogenates used in HL activity determination was performed by the method of Hansson et al (21) which was partly modified in our laboratory: The livers stored at -20 °C were thawed and about 1.0 gram liver tissue was washed three times with ice-cold 154 mM NaCI. The tissues suspended in 5 ml cold Krebs Ringer/Hepes buffer (pH: 7.4) were homogenized. Homogenate was centrifuged at 4000 x g for 15 min at 4 °C. The supernatant, containing the floating fat and cytosol fractions, was discarded. Microsomal fraction was suspended in 5.0 **ml** of cold 0.1 M Tris buffer (pH: 9.0) containing 15 IU of heparin, and the incubated for 45 min in an ice-bath and mixed with homogenizator for 15 min intervals during incubation time. After centrifugation, supernatant was used in HL measurement.

HL activities were assayed by using the titrimetric Sigma-800 kit for serum lipase activity. In addition, protein content of supernatants were measured by Lowry Method (22), and HL activity was expressed in units per miligram of protein as specific activity (mU/mg protein).

Statistical Analysis: Statistical evaluation of difference between means were determined by Student's t test (23).

RESULTS

Because one rat of short-term LD EE/LNG group died during the experiment period, the study was completed with 61 rats. The plasma TG, HDL-chol, LDL-chol, Apo A1 and B values and HL activities of study groups were given in Table 1.

Table 1. Biochemical parameter values measured in study groups

	Short-term (15days)			Long-term (120 days)		
	Control (n=10)	LD EE/LNG (n=9)	HD EE/LNG (n=10)	Control (n=10)	LDEE/LNG (n-11)	HD EE/LNG (n-11)
TG (mg/dl)	37.2±7.23	d 47.4±0.94	a, ** 66.0±15.15	36.3±5.39	41.0±7.82	a,** 62.54±14.16
HDL-chol (mg/dl)	43.13±5.07	40.34±7.03	a, 33.27±5.35	45.59±7.64	45.67+5.74	a, 31.16±4.39
LDL-chol (mg/dl)	16.96±6.52	d 23.77±7.38	a,* 43.80±9.20	17,23±6.09	d 24.47±7.52	a,*,AAA 59.26±13.02
Apo Ai (mg/dl)	37.04±7.66	41.48±4.08	c,** 46.13±5.09	42.81 ±6.26	d.AA 50.87±6.79	d,*,A 35.17±7.87
Apo B (mg/dl)	18.96±6.56	19.13±3.87	22.47±5.77	22.68±6.71	23.76±6.43	24.77±7.49
HL (mU/mg prot.)	69.07±32.43	b 28.56±11.31	a 24.39±9.36	67.47±28.82	b 33.37±12.51	d,*,A 99.58±25.59

The values were expressed as means ± standard deviation (X+SD)
Stastical comparison of EE/LNG groups were made according to:
control groups (a: p<0.001; b: p<0.005; c: p<0.01; d: p<0.05)
- dose (LD vs HD) (*: p<0.001; ": p<0.05)
- treatment-duration (short-term vs long-term) (A: p<0.001; AA: p<0.005; AAA: p<0.01)
n: The number of rats in each group
LD: low-dose; HD: high-dose

When compared to controls, the increase in plasma TG levels, except in long-term LD EE/LNG group, was found to be important ($p<0.001$; $p<0.05$). The effect of EE/LNG combination on TG seems to be dose-dependent in both short and long term studies. In all study groups, plasma LDL-chol levels were higher than in controls ($p<0.001$;$p<0.05$). EE/LNG increased LDL-chol levels depending on dose ($p<0.001$); but the effect of treatment-duration on LDL-chol was seen at high doses($p<0.01$). When HDL-chol levels were compared to controls, HDL-chol was not changed statistical in LD EE/LNG groups ($p>0.05$); but significantly decreased in HD groups ($p<0.001$). EE/LNG affected HDL-chol depending on the dose ($p<0.001$; $p<0.05$). In short-term study groups, the increase in plasma Apo A1 levels was only important in HD EE/LNG group ($p<0.01$). In long-term, EE/LNG increased Apo A1 levels at low doses ($p<0.05$), but decrease at high doses ($p<0.05$). It was found that EE/LNG affected ApoA1 by both dose ($p<0.05$; $p<0.001$) and time ($p<0.005$; $p<0.01$). On the other hand, plasma Apo B levels were not changed during the study.

When compared to controls, HL activities were increased in long term HD EE/LNG group ($p<0.05$), but decreased in other groups ($p<0.001$; $p<0.005$). EE/LNG combination decreased enzyme activity at low doses by independing on time, but affected at high doses by depending on time ($p<0.001$).

DISCUSSION

In additional to their central actions in the hypothalamo-pituitary axis, steroid hormones have direct effect on peripheral metabolic tissues. The liver, among extragenital organs, is te principal one affected by sex steroids (24). It has been assumed that sex steriods exert their effects by influencing synthesis and/or turnover of lipoproteins by the liver (25). Experiments have indicated that about 80% of LDL removed from the blood is internalized by the liver (26), which has most of LDL-receptors (27). In vivo (28) and in vitro (27) studies have suggested that EE administration at pharmacologic doses induces hypolipidemia by increasing the number of LDL-receptors in livers of rats. On the other hand, Tkocz et al (29,30) have observed that the administration of EE at low doses to female rats elevates plasma T.chol and LDL-, VLDL-chol levels, and suggested that the increase in LDL-chol may be a consequence of increased VLDL production, because a precursor-product relationship seems to exist between VLDL and LDL. Although it is known that progestins generally trend to increase in LDL-chol levels, there are many studies which opposite to this: It is shown that the progestin, LNG, administrated orally to fed female rats significantly rises plasma LDL-chol levels (8). In contrast, acut treatment with estrane type progestins significantly lower LDL-chol in rats dose-dependently (29,30). In addition to animal studies, the data from clinical trials have shown that EE/LNG combination increase (11,31) or not change (12,14) plasma LDL-chol levels.

In our study, EE/LNG combination given rats increased plasma LDL-chol levels depending on dose and time. These findings do not completely agree with those of data from animal studies. However, the dose, treatment-duration and route of sex sterids in these animal studies are the factors which may affect the results. In addition, if it is accepted that combined steroids used for rats are estrogen-dominant (32), the findings of Tkocz et al (29,30) are in agreement with our results. Moreover, according to the most of authors, the principal effect cf progestins used in combinations seems to depend on dose and intrinsic androgenic properties. Since LNG, among 19-norprogestins, has the most androgenic, antiestrogenic and progestational activities, may be more effective on lipids (11,12). From this point of view, it might be expected that EE/LNG combination increases LDL-chol levels.

TGs are transported through the blood stream mainly by chylomicrons and VLDL. Although it is known that estrogens increase hepatic synthesis and secretion of VLDL-TG (3,33), it is reported that the administration of EE at low doses elevates plasma TG levels (34,35) and opposite to this, HD of EE produces a profound hipolipidemia in rats (36). Reports concerning the effects of progestins on TG are also conflicting. It has been suggested that LNG decreases (8,37), or not change plasma TG levels, and also stimulates hepatic TG release (35).

In the present study, EE/LNG increased TG levels depending on dose, fince we could not find a previous report, it was not possible to compare our findingswith the literature. However, there are many clinical studies wihch show EE/LNG combination increases plasma TG levels (11,31,38).

It is known for a long time there is an inverse relationship between plasma HDL-chol and the development of cardiovascular disease (CVD) (39). Moreover, the HDL-2-subfraction is found to be better discriminator, for the risk of CVD (10). Sex steroid hormones have been suggested to play an important role in regulating HDL levels in plasma, and HDL-chol levels are higher in females than in males after puberty. It has been reported that the administration of EE to humans (3,40), rats (29,30) and rabbits(41) increases plasma HDL-chol levels. On the other hand, it has been shown that LNG does not change (8), or lowers HDL-chol in rat plasma; further, EE stimulated increase of HDL-chol is found to be suppressed by these progestins (29,30). In addition, in the studies on monkeys, it is seen that EE/LNG (42) and EE/NG (43) combinations decrease HDL-chol.

In thepresent study, it was observed that EE/LNG combination at low doses did not statisticaly change HDL-chol levels; but significantly decreased at high doses. In spite of differences in study protocols, studies on monkeys (42,43) and rats (29,30) showing

EE/LNG or EE/NG combinations, and LNG used with EE sequentially decrease plasma HDL-chol levels are in agreement with our findings.

Apolipoproteins are also another fraction which play an important role in lipoprotein metabolism. The concentrations of Apo A1 and B, the major protein contents of HDL and LDL respectively, may be a better discriminator for CVD risk than those of lipoprotein-cholesterols (44). Schaefer et al(3) who investigate the effects of EE on Apo A1 and B in premenoposal women have shown that estrogen treatment stimulates VLDL-Apo B production and HDL-ApoA1 synthesis with no change in LDL-Apo B synthesis. The findings of Kuswaha et al (45) on Apo A1 synthesis and catabolism in baboons treated with estrogen alone or in combination with progesterone are also consistent with these observations. On the other hand, in their another study on Apo B levels in baboons have been reported that the decrease in LDL-Apo B levels is parallel to LDL-chol in estrogen-and estrogen/progestin-treated baboons (46).

Te effect of exogenous estrogens on plasma Apo A1 and B levels seem to be primarily dose-dependent (25,36). Weinstein et al have reported that plasma Apo A1 levels are increased with the treatment of EE at low doses (47), but decreased at high doses (36) in rats. Tarn et al (25) have suggested that lipid responses to estrogen treatment may be mediated partly via specific hormonal binding sites in the liver, and using an in vitro model, shown that estrogen at low doses leads to maximal increases in Apo A1 secretion via induction of nuclear type 1 estrogen binding sites, while smilar increases in Apo B synthesis in response to high concentrations of hormone with the induction of type II sites.

In our study, EE/LNG combination affected Apo A1 levels by dose and treatment-duration; but did not Apo B. EE/LNG combination at low doses did not change Apo A1 levels in short term, but by the time, increased Apo A1 significantly. On the other hand, the high doses of EE/LNG which increase Apo A1 levels in short-term decreased Apo A1 levels when used for long-term. When it is taken into consideration that the combination used in the present study is estrogen dominant (32), and even in the present of the differences in study protocols, our findings are in agreement with the reports showing LD EE increases (3,25,45,47) and HD EE decreases (36) Apo A1 levels. In addition, Luoma et al (48) have indicated that estrogen-dominant EE/LNG combination elevates HDL-chol and Apo A1 levels causing a decrease in HL activity. According to kinetic hypothesis of Kuswaha et al (45,46), it is possible that the antagonistic effects of estrogens and progestins on Apo A1 and B may be mediated by HL. As it will be discussed later, the changes in plasma Apo A1 levels and HL activities in our study are also taken into account together.

HL plays an important role in lipoprotein metabolism, which participates in the catabolism of several lipoprotein classes and lipoprotein interconversions (49,50). In recent years, a negative relationship between plasma HDL, especially HDL2 concentration and postheparin plasma HL activity, and TG-rich HDL2 can be converted to HDL3 by HL, have been reported (51).

It is known that HL is modulated by both endogenous and exogenous sex steriods (52). Estrogens increase the levels of HDL-chol, HDL-TG, HDL-Apo A1, HDL2-chol, and HDL2-PL by reducing postheparin plasma HL activity (3,53), whereas progestins derived from 19-nortestosterone decrease these lipoprotein constituents by incerasing HL activity and therefore may counteract the estrogent effect (52,53). In the study of Leuven et al (54) which investigated the effect of EE/LNG combination on HL in women, no significant changes in HDL levels but a decrease in HL activity have been shown and have been concluded that the effects of two components of combination on HDL are almost in balance; but that HL-reducing effect of estrogen component dominates.

In the present study, the effect of EE/LNG combination on HL activity was not time dependent at low doses, but time-dependent at high doses. Because, the decreased activity by using HD combination in short-period was significantly increased by the time. Since we could not find any -study which shows the effect of E/P combinations on HL activity by using an animal model, it was not possible to compare our results with the literature. However, the findings of Leuven et al (54) support our results.

When taken into account all these observations of the present study; in LD EE/LNG groups, the finding of the increase or trended to increase in TG and Apo A1 levels depending on the dose, may be partly explained by the finding that HL activities are decreased. Since there was also no an important change in HDL-chol and Apo B values in this group, it might be thought there was a balance in between EE and LNG components of combination; but that HL-reducing effect of estrogen component dominates.

In short-term HD EE/LNG group, increased TG and Apo A1 levels and decreased HL activity may be explained by estrogenic activity; whereas decreased HDL-chol levels may be attributed to the antiestrogenic effect of LNG and impraired balance between components. On the other hand, the long-term administration of EE/LNG at high doses leads to more increase in HL activity and significantly decrease Apo A1 and HDL-chol levels. It might be thought progestin component of combination dominates. Since LNG, among 19-norprogestins, has the most potent biological activity, it may be expected that LNG, used for long term at high doses, clearly shows its andorogenic/antiestrogenic effects in this group. But it is not possible to explain the findings that the increase in TG depending on LNG effect and current literature knowledge.

However, La Rosa (55) has suggested that incerases in T.chol and TG levels are not progestin related but may be considered specific to estrogen component of contraceptive steroids. In addition, increases in LDL-chol and decreases in HDL-chol and Apo A1 levels appear to be dependent on the type of progestin, with the most androgenically potent agent such as LNG (55).

In addition, when the data from the first and second parts of the present study are taken into consideration together, it might be thought the observed changes in enzyme levels (such as HMG-CoA synthase, AcAc-CoA thiolase and HL) may lead to the changes in plasma lipids and lipoproteins as noted clinically with the use of contraceptive steroids and the changes in hepatic cholesterol and lipoprotein metabolism as a consequence. With the use of EE/LNG combination, the suppression in HL activity opposite to increase in enzyme activities related with cholesterol synthesis are important finding at cellular level. The inhibition of HL, completes the reverse transport cycle of cholesterol from peripheral tissues to the liver, will lead to impairment of the flux of cholesterol to the liver and therefore de novo cholesterol synthesis will increase. Indeed, it has been shown that the inhibition of HL in vivo leads to induction of de novo cholesterol synthesis in rat liver and also affects all plasma lipoprotein fractions (49).

In conclusion, although the current literature describing the effects of contraceptive steroids on lipoprotein metabolism not entirely consistent and animal studies which include high doses of steroids do not fully represent the clinical situation, the influence of contraceptive steroids on plasma lipoprotein metabolism in both rats and humans is an important finding. When clinical correlation of our findings from rat model is made, it has been suggested that decreasing the doses in E/P combinations, changing the component ratios to reach a physiological state, selecting the progestins having less androgenic activities as well as administrating for short periods may modify or remove the effects of these hormonal contraceptive agents on plasma lipoprotein metabolism. In addition, since the individual metabolic response to these combinations varies, it may be useful that the lipid,lipoprotein and apolipoprotein profiles, and postheparin plasma HL activity of the women should be monitored with certain intervals.

Etinil estradiol/levonorgestrel (EE/LNG) kombinasyonunun dişi ratlarda plazma lipoprotein metabolizması üzerine etkisi (II)

Bu çalışma, EE/LNG kombinasyonunun, plazma lipoprotein metabolizması üzerine doza ve süreye bağlı etkilerini incelemek amacıyla yapıldı. Kısa ve uzun süreli çalışmalar için 2 gruba ayrılan ratlar, daha sonra düşük doz (DD) ve yüksek doz (YD) EE/LNG verilen gruplar ve kontrol grubu olmak zere üç gruba bölündü. Deney sürelerinin sonunda, ratların plazmasında trigliserid (TG), LDL-kolesterol (kol), HÜL-kol, apolipoprotein (Apo) Aı ve B seviyeleri ve karaciğerlerinde hepatik lipaz (HL) aktivitesi tayin edildi.

Kontrollerle karşılaşıldığında , tüm gruplarda plazma LDL-kol seviyeleri daha yüksek bulunurken, uzun süre DD EE/LNG grubu dışında TG seviyeleri de yükseldi. Buna karşılık YD gruplarında plazma HDL-kol seviyeleri asaldı. Kısa süre YD EE/LNG grubunda plazma Apo Al yükselirken, uzun süreli çalışmada DD'da uygulanan EE/LNG, Apo A1'i artırdı fakat YD'da düşürdü. Plazma Apo B seviyeleri ise tüm gruplarda istatistik/" bakımdan değişmedi. HL aktivitesi, uzun süre YD EE/LNG grubunda yükselirken, diğer gruplarda azaldı. Çalışma grupları doza ve süreye göre karşılaştırıldığında, EE/LNG 'nin TG ve HLD-kol'ü doza bağlı; HL, LDL-kol ve Apo AVİ doza ve süreye bağlı olarak değiştirdiği gözlendi.

Sonuç olarak, EE/LNG kombinasyonunun plazma lipoprotein metabolizması üzerindeki etkilerinin. HL aktivitesinde gözlenen değişikliklerle ilişkili olabileceğ kanaatine varıldı. ʻTurk J Med Res 1993; 11 (6): 266-272]

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