The effect of ethinyl estradiol/levonorgestrel (EE/LNG) combination on hepatic cholesterol synthesis in female rats (I)

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In order to investigate dose-and time-dependent effects of EE/LNG on hepatic cholesterol synthesis; rats were divided into 2 groups forshort-and long-term investigations. Then each group was subdivided into 3 subgroups according to orally given low-dose (LD) and high-dose (HD) of EE/LNG combinations and control group. At the end of the experiment periods, total cholesterol (T.chol) levels In plasma and hydroxy methylglutaryl-Coenzyme A (HMG-CoA) synthase and acetoacetyl-Coenzyme A (AcAc-CoA) thiolase activities in the liver of rats were measured.

Plasma T.chol levels were found to be higher in HD EE/LNG groups than in controls; but there was no significant change in T.chol levels in LD group during experiment period. In short-term investigation, EE/LNG treated at HD increased both enzyme activities more than controls, whereas HMG-CoA synthase was only increased by LD. On the other hand, enzyme activities were either decreased or not changed in long period.

These findings were suggested that LD EE/LNG has no significant effect on hepatic cholesterol synthesis, but increased enzyme activities by HD in short period may increase plasma T.chol levels, and by the time, these rising cholesterol levels may suppress enzyme activit, by inhibition. [Turk J Med Res 1993; 11(6):261-265]

Key Words: Hidroxymethyl glutaryl CoA synthetase, Rats, Estradiol

Numerous epidemiologic studies have demonstrated a relationship between contraceptive steroid use and cardiovascular disease (1,2,3). However, the current literature describing the effect of contraceptive steroids on lipid metabolism is not entirely consistent (4,5,6). Because, combined contraceptive steroids may change lipid metabolism depending on the dose of both estrogen and progestogen components, and on the type of the progestational agent as well as on the treatment duration and the route of administration of the preparation used (7,8). In recent years, even when very low-dose (LD) combinations were used, some alterations in lipid metabolism which were in minimal levels have been observed (9,10,11). For this reason, it will be necessary to gather more detailed metabolic studies in cellular enzyme levels for explaining the mechanism of the interactions of estrogens and progestins.

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The purpose of this study is to determine the effect of EE/LNG combination which orally given on hepatic cholesterol synthesis depending on the dose and the treatment-duration, in the rat model. For this reason, total cholesterol (T.chol) level in plasma samples and hydroxymethyl glutaryl-Coenzyme A (HMG-CoA) synthase and acetoacetyl-Coenzyme A (AcAc, CoA) tiolase activities in the livers of female rats treated with low-and high-dose EE/LNG combination for short (15 days)-and long (120 days)-terms were measured.

MATERIALS AND METHODS

Material: Estrogen/progestin (E/P) combination containing 0.03 mg EE/0.125 mg LNG in each pill was selected from commercial preparations. All chemicals used in biochemical measurements were of analytical grade.

Animals: Mature, 75-85 day old, female Swiss albino rats weighing 140-180 grams were used for all groups. During the experiment period; the rats were kept at normal room temparature and humidity, were fed Standard Purina Rat Chow and tap water ad libitum.

Experimental Design: A total of 62 female rats were divided into 2 groups for short (15 days)-and long (120 days)-term investigations, and each group was then subdivided into 3 subgroups according to orally given low-and high-doses of EE/LNG combinations and control group (Table 1).

Dose adjustment of EE/LNG combination was performed according to the literature describing "low-and high-dose" terms for animal experiments: "The low-dose is usually set at 2-5 times the anticipated clinical dose on a milligram per kilogram of body weight. The high dose for rats usualy ranges from 200-400 and occasionally up to 1000 times the anticipated clinical dose" (12). Therefore, during the experiment period, the choice of dose in rats was performed according to 4 times for low-dose (LD) and 200 times for high-dose (HD) anticipated clinical dose on a milligram per kilogram of body weight (Table 1).

As it is planned to add the combinations which would be given orally to rats' water; prior to experiments in order to determine daily water consumption of rats, water-test was performed for three days. At the end of the test, the mean water consumption of each rat was found to be 25 ml.

For the low-and high-dose of EE/LNG combinations, necessary calculations were performed on the mean weights of rats in cages in five or six groups. The daily doses were added to the water supply for each cage. For short (15 days)-and long (120 days)-terms study groups, the same processes were repeated every day at 9.00 a.m. In addition, throughout the experiment period, the rats were weighed every week and dose adjustments were performed on the mean weights of the rats.

At the end of the experiment periods, rats were fasted overnight. The following morning, the rats' carotid arteries were cut under anaesthesia with diethyl ether and blood was taken in tubes containing EDTA (1.5 mg/ml blood). Plasma was seperated by centrifugation at 1500xg at 4°C for 20 minutes. Plasma T.chol levels were determined by modified Leffler method (13) on the same day. The livers removed from rats were washed three times with ice-cold 154 mM NaCl, and stored at -20°C until enzyme analysis (14).

The preparation of Cytoplasmic Fraction from Liver Homogenates (15): The livers stored at 20°C were thawed and cut into small cubes and washed with ice-

Table 1. Study design of rats.

GROUPS	n	Short-Term (15 days) (EE/LNG) dose treated (^g/kg rat weight/day)	n	Long-Term (120 days) (EE/LNG) dose treated (ng/kg rat weight/day)
Control	10		1U	
LDEE/LNG	10	(2.4/10)	11	(2.4/10)
HDEE/LNG	10	(120/500)	11	(120/500)

n: The number of rats in each group

Table 2. Biochemical parameter values measured in study groups.

	Short-Term			Long Term		
	CONTROL (n=10)	LD EE/LNG (n=9)	HD EE/LNG (n=10)	CONTROL (n-10)	LD EE/LNG (n=11)	HD EE/LNG (n=11)
T. Chol (mg/dl)	65.40±10.41	70.88±11.68	a, *** 86.5+12.50	68.0+12.36	76.0±12.13	a, *, AAA 99.36± 15.52
HMG-CoA synthase (mU/mg prot.)	1.979+0.356	c 2.483±0.462	b 2.624±0.460	2.164±0.325	2.231+0.586	AA 1.780±0.464
AcAc-CoA thiolase (mU/mg prot.)	226.44±25.85	223.34±42.10	b," 293.68±53.83	227.39±38.53	213.59+48.20	b,"*, A 164.82±36.21

The values are expressed as means ± SD
Statistical comparison of EE/LNG groups were made according to:
- Control groups (a: p<0.001; b: p<0.005; c: p<0.05)
- Dose in themselves (LD vs HD) (*: p<0.001; **: p<0.01; ***:p<0.05)
- Treatment-duration (short-them groups vs long-term groups): (A: p<0.001; AA: p<0.005; AAA: p<0.05)
n: The number of rats in each group.

cold homogenizing buffer containing 0.25 M sucrose, 0.1 mM EDTA, and 2.0 mM Hepes, pH:7.2. Then one gram of cubed liver was suspended in 5 ml of homogenizing buffer and then homogezined by three passes of a motor-driven Teflon pestle in loose fitting homogenizer. Homogenate was centrifugated at 11.300xg for 15 minutes. The supernatant was collected as cytoplasmic fraction. Triton x-100 was added to supernatant as a final concentration of 0.2% (w/v). This mixture stored in ice-bath was used for enzyme analysis on the same day.

Enzyme Analysis: HMG-CoA synthase (EC. 4.1.3.5) activity was determined by using a spectrophotometric assay which measures the acetyl-CoA dependent dissappearance of AcAc-CoA at 300 nm (14,15). AcAc thiolase (EC. 2.3.1.9) activity was measured according to the spectrophotometric condensation method, modified by Clinkenbeard et al (16). In both assays, one unit of enzyme activity was defined as the formation of 1 umol HMG-CoA and AcAc-CoA per minute for synthase and thiolase, respectively (14). The protein contens of supernatant was measured by Lowry method (17), and enzyme activities were expressed in units per milligram of protein as specific activity (mU/mg protein).

Statistics: Student's t test was used to evaluate the significance of the data obtained (18).

RESULTS

Because one rat of short-term LD EE/LNG group died during the experiment period, the study was completed with 61 rats. Biochemical parameter values measured in groups were shown in Table 2.

Plasma T.chol levels were found to be higher in HD EE/LNG groups than in controls ($p<0.001$); but there was no significant change in T chol levels in LD group ($p>0.05$) during experiment period. The effect of EE/LNG combination on T chol increased depending on dose ($p<0.001$; $p<0.05$), and treatment duration was also important at HD EE/LNG group.

When compared to controls, HMG-CoA synthase activity was increased in short-term groups ($p<0.005$; $p<0.05$); but not changed in long-term groups ($p>0.057$). On the other hand, AcAc-CoA tiolase activity increased in short-term HD EE/LNG group ($p<0.05$) was significantly decreased in long period ($p<0.005$). It has been seen that HD EE/LNG combination effected HMG-CoA synthase activity depending on treatment duration ($p<0.005$). The changes observed in AcAc-CoA thiolase was both dose ($p<0.01$; $p<0.05$)-and time ($p<0.001$)-dependent.

DISCUSSION

In the present study, dose-and time-dependent effects of EE/LNG combination which orally given on hepatic cholesterol synthesis were investigated at cellular level in female rats. Compared to the 30 day cycle in humans, the rat has a very short estrus cycle of 4-5 days. This results in an overlap of the follicular and luteal phases (12). Therefore, in order to compare the animal experiment accepted as preclinic study with clinic studies, short-term investigations carried on for 3 months and long-term investigations carried on for 2 years clinically were assumed to be equal to 15 days and 120 days in rats, respectively.

In the present study, it has been found that EE/LNG combination increased plasma T chol levels by dose and treatment-duration was also an important factor at high dose. Since we could not find a previous report concerning the combined effects of EE and progestin used as in this investigation on plasma T chol levels, it was not possible to compare our results with the literature related to animal studies. However, there are contraversial reports about estrogens administrated to rats as a decrease (19,20), or an increase (21,22) in plasma T chol levels. In addition, the administration of estrogens alone induce dose-dependent changes in plasma T chol levels of rats (23). Reports concerning the effects of progestins are also conflicting. It has been reported that LNG increased plasma T chol levels (24) or NEA (norethisteron acetat) and NG (norgestrel) decreased T chol (22). The dose, the type, treatment-duration and route of sex steroids used in animal studies are the factors which may affect the results. In addition, it is known that the effect of synthetic entrogens on lowering plasma T chol is rather weak than natural ones (25). On the other hand, experience has shown that effects of progestins differ from one species another. Progestins, the 19-nortestosteron derivaties, having strong progestational activity in the human, are usually weakly progestational in the dogs and rats (12,26).

If it is accepted that contraceptive steroids given as multiple doses of clinical dose in rat studies are estrogen-dominant (27), our findings agree with that of Tkocz (21,22) and Ferreri (23) et al. Although the preparations used in this study may accept as estrogen-dominant, there may be a synergic interaction between the estrogen and progesr.n components of combination, such as noted for contraception. For this reason, it may be excepted that EE/LNG combination used for long-time at high-doses can cause more increase in T chol levels.

The formation of HMG-CoA and mevalonate from acetyl-CoA units in cholesterol synthesis are catalyzed by the actions of 3 enzymes: AcAc-CoA thiolase, HMG-CoA synthase and HMG-CoA reductase (28). The regulation of cholesterol synthesis is mediated by the coordinate regulation of several enzymes in the isoprenoid biosynthetic pathway (29). But the nexus of primary control appears to be at the level of HMG-CoA; thus, the regulation of HMG-CoA synthase and AcAc-CoA thiolase activities would appear important as well as HMG-CoA reductase (30).

In vivo studies using rat model have shown that cholesterol feeding and starvation decrease endogen cholesterol synthesis by suppressing the activities of of HMG-CoA reductase (29,30), HMG-CoA synthase (30,31) and AcAc-CoA thiolase (16,32); but cholestyramine feeding promotes an increase in hepatic cholesterogenesis by enhancing all of three enzymes activities (29,30,32). In addition, it has been shown that cytoplasmic HMG-CoA synthase regulation occurs primarily at the level of mRNA and coordinate with HMG-CoA reductase (29) as well as AcAc-CoA thiolase (33).

In our study, it has also been observed that HMG-CoA synthase and AcAc-CoA thiolase activities are almost changed in parallel by E/P combination. In short term investigation, HMG-CoA synthase activity was increased in both LD-and HD EE/LNG groups; whereas AcAc-CoA thiolase activity was increased in HD group. In long periods, there was no change in enzyme activities of LD group; but a significant decrease in HD EE/LNG group.

To our current knowledge, the present study is the first evaluate the effects of EE/LNG combination on hepatic HMG/CoA synthase and thiolase activities. However, Letterie et al (34) observed a significant increase, independent dose, in hepatic HMG-CoA reductase activity in their study where female rats were treated with low and high-doses of EE, NG and EE/NG combinations, and suggested that there was a synergic interaction between EE and 19-norprogestins which lead to the induction in HMG-CoA reductase activity (34). When it is taken into consideration that the data concerning HMG-CoA reductase, synthase and thiolase are regulated together as described earlier (29,32,33), the present findings about EE/LNG at low- and hihg-doses increases enzyme activities in short period are in agreement with the results of Letterie et al (34). That is, the increase in reductase activity with E/P combinations supports the concern about HMG-CoA synthase and AcAc-CoA thiolase regulated together with reductase may be increased by conraceptive steriods.

In short term study, the changes in enzyme activities seem to be reflected on plasma T. chol levels. Because, high T. chol levels accompany high enzyme activities in HD EE/LNG group, and the parameters, except HMG-CoA synthase, were not changed in LD group. In long-term study, there was also no change in T chol and enzyme activities at low-doses, but a significant increase in T chol and a significant decrease in enzyme activities at high doses EE/LNG.

These findings were suggested that LD EE/LNG has no significant effects on hepatic cholesterol synthesis, but increased enzyme activities by HD in short-period may increase plasma T chol levels, and by the time, these rising cholesterol levels may suppress enzyme activities by inhibition.

Etinil estradiol/levonorgestrel (EE/LNG) kombinasyonunun, dişi ratlarda hepatik kolesterol sentezi üzerine etkisi (I)

EE/LNG kombinasyonunun hepatik kolesterol metabolizması üzerine, doza ve süreye bağlı etkilerini incelemek amacıyla yapılan bu çalışmada, ratlar kısa ve uzun süreli çalışma için 2 gruba ayrıldıktan sonra, her grup kontrol, düşük doz (D D) ve yüksek doz (YD) EE/LNG verilen gruplar şeklinde 3 alt gruba bölündü. Deney süreleri tamamlanan rafların plazmasında total kolesterol (T. kol) seviyeleri ve karaciğerllerinde hidroksi-metilglutaril-Koenzim A (HMG-KoA) sentaz ve asetoasetil-Koenzirn A (AcAc-KoA) tiyolaz aktiviteleri ölçüldü.

Çalışma süresince, YD EE/LNG gruplarında plazma T. kol seviyeleri kontrollere göre daha yüksek bulunurken; DD EE/LNG gruplarında değişmedi. Kısa süreli çalışmada YD'da uygulanan EE/LNG, kontrole göre, her iki enzim aktivitesini de artırırken; DD'da sadece HMG-KoA sentaz yükseldi. Uzun süreli çalışmada ise, enzim aktiviteleri ya azaldı, ya da değişmedi.

Bu bulgular, DD'da uygulanan EE/LNG'nin hepatik kolesterol metabolizmasına önemli bir etkisi olmadığı; fakat YD EE/LNG ile kısa sürede yükselen enzim aktivitelerinin plazma T. kol seviyelerini artırdığı ve sürenin ilerlemesiyle gittikçe yükselen plazma T. kol seviyelerinin, inhibisyon yoluyla enzim aktiv itelerin i baskılayabileceği şeklinde yorumlandı. [TurkJ Med Res 1993; 11(6): 261-265]

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