Effect of synthetic oestrogens and progestagens in oral contraceptives on bile lipid composition

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SUMMARY The prevalence of cholesterol gall stones in young women has increased since the introduction of oral contraceptives. The synthetic female sex hormones used in these preparations, increase the degree of cholesterol saturation in bile. To determine whether oestrogens, progestagens, or both, are responsible for the change in biliary cholesterol saturation index, a prospective randomised, controlled study was performed. A significant increase in the cholesterol saturation index of bile was observed when either 30 μg ethinylestradiol plus 150 μg norgestrel (p=0.01) or 50 μg ethinylestradiol plus 250 μg norgestrel (p<0.01) were ingested daily for two months. No change in the cholesterol saturation index was observed when 30 μg ethinylestradiol alone, or 30 μg ethinylestradiol plus 2.5 mg norethisterone were used. The mechanism for the increase in cholesterol saturation index did not appear to involve bile acid metabolism. These results indicate that the progestagen, norgestrel, and not as previously thought the oestrogen, ethinylestradiol, is responsible for the increase in cholesterol saturation of bile which accompanies the use of oral contraceptives.

Epidemiological studies have suggested that there must be a causal relationship between female sex hormones and gall-stone disease. Gall stones are twice as common in females once puberty has been reached. After the menopause this sex difference gradually diminishes. Similarly, pregnancy and the use of synthetic female sex hormones for contraception or the prevention of postmenopausal symptoms have been shown to increase the incidence of gall-stone disease.

Approximately 85% of gall stones are composed predominantly of cholesterol. It is now recognised that cholesterol gall stones will form only when bile is supersaturated with cholesterol. Conversely, dissolution of cholesterol gall stones may occur when bile becomes unsaturated with cholesterol. In 1973 Thomas and Hofmann introduced the cholesterol saturation index (CSI) to quantify the degree of cholesterol saturation in bile. Any substance or factor that increases the cholesterol saturation index of bile may alter the relative time periods of cholesterol unsaturation to supersaturation during the day in favour of cholesterol supersaturation within the gall bladder and lead to cholesterol crystal formation and gall-stone growth.

Studies have been performed in reproductive women to investigate the effect of female sex hormones on the cholesterol saturation index of bile. Physiological changes in natural sex hormones during a normal menstrual cycle have been shown to have no effect in four separate studies, and a small effect in a study by Low-Beer et al. The withdrawal of natural female sex hormones by oophorectomy in one woman reduced the cholesterol saturation index of bile. The ingestion of synthetic female sex hormones in oral contraceptive doses has been shown to increase both the cholesterol saturation index of bile and the proportion of women with supersaturated (CSI >1.0) bile. It has been claimed that oestrogen is responsible for these changes. The evidence, however, is circumstantial. Studies in rats, monkeys, and baboons which used oestrogen doses up to 1000 times per unit weight greater than that used in oral contraceptives have shown that oestrogens can alter the lipid composition and the
bile acid kinetics of bile, but not always in favour of cholesterol supersaturation. Clearly, species differences exist. We therefore decided to investigate in humans, firstly, whether contraceptive doses of synthetic oestrogens or progestagens were responsible for the reported rise in the biliary cholesterol saturation index and, secondly, whether this could be attributed to an effect on bile acid metabolism.

Methods

The original objective was to evaluate the effect on the biliary cholesterol saturation index of equivalent pharmacological doses of the synthetic female sex hormones commonly used in oral contraceptives: 30 μg ethinylestradiol alone, 150 μg norgestrel alone, 30 μg ethinylestradiol plus 150 μg norgestrel, and 30 μg ethinylestradiol plus 2.5 mg norethisterone. Fears were raised about possible side effects from the use of 150 μg norgestrel alone because this dose was five times that currently used in progestagen alone oral contraceptives. A brief pilot trial showed that the use of this dose of norgestrel alone for longer than one month caused persistent nausea and breakthrough uterine bleeding. Therefore, the part of the study dealing with norgestrel alone had to be replaced. A ‘high’ oestrogen dose oral contraceptive, 50 μg ethinylestradiol plus 250 μg norgestrel, was chosen because most of the oral contraceptives used in the original study of Bennion et al contained 50 μg ethinylestradiol.

We undertook a prospective controlled trial in 10 healthy female volunteers, aged 19 to 35 years, known to have normal oral cholecystograms. Intravenous contraceptive devices were used as necessary to prevent pregnancy. The study was approved by the Hospital’s ethics committee.

The design of the study was as follows: there were five phases – a physiological control menstrual cycle and four oestrogen/progestagen phases. The order of participation in each phase was randomised. In each oestrogen/progestagen phase, the prescribed dose (see above) of the synthetic female sex hormone(s) was ingested daily from the fifth to the 26th day of the 28-day cycle for two cycles. When a control menstrual cycle followed an oestrogen/progestagen phase, two physiological menses were allowed to elapse before bile sampling to ensure complete elimination of the synthetic hormones and allow ovulation to start again. Gall-bladder bile and blood samples were taken on the 23rd and 26th day of the second month in each of the oestrogen/progestagen phases, and on the third, 13th, and 21st day of the control menstrual cycle to measure the biliary cholesterol saturation index, the bile acid profile, the total bile acid pool size, and the level of circulating serum progesterone.

Bile sampling

Duodenal collections of gall-bladder bile were made exactly 12 hours after the ingestion of a standard meal—two slices of bread and butter and a glass of milk. The subjects swallowed an AN-20 weighted duodenal tube (Anderson Products Inc. Oyster Bay, New York, USA). Gall-bladder bile samples were aspirated from the duodenum after a slow intravenous injection of 1 U/kg Pancreozymin (Boots Co Ltd, Nottingham, England). A 2 ml sample of the most concentrated bile was immediately sent to the laboratory for lipid analysis. Excess bile was returned to the duodenum to avoid depletion of the bile acid pool.

Bile lipid analysis

The biliary lipids (cholesterol, total bile acids, and phospholipids) were assayed using fresh bile for solvent extraction as described by Whiting et al. Assays were performed in several batches as samples became available during the study. The cholesterol saturation index was calculated by the method of Thomas and Hofmann using the cholesterol equilibrium saturation limit of Holzbach et al.

Individual bile acids in the gall-bladder bile samples were deconjugated and quantified by gas liquid chromatography as described by Whiting and Watts. Individual bile acid profiles were determined by expressing the relative concentrations of the individual bile acids as moles per cent.

Bile acid pool size

The total bile acid pool size was measured by the simplified isotope dilution technique of Duane et al. Two minor modifications were used. Firstly, the 10 ml intravenous injection of radioactive cholate was always given half an hour before eating the standard evening meal. Secondly, the addition of non-radioactive sodium cholate to the vial of radioactive cholic acid proved essential to prevent major losses from the solution of radioactive cholate by absorption to glass surfaces. 14C-carboxyl cholic acid (specific activity 50-60 mCi/mol; the Radio Chemical Centre, Amersham, England) and (2,4-3H) cholic acid (specific activity 14 Ci/mmol; New England Nuclear, Boston, USA) were purchased as sterile solutions in ethanol. Each isotope was repackaged in a 100 ml sterile injection vial of 0.9% sodium chloride with 175 meq/l sodium bicarbonate, 0.2 mg/ml cold carrier sodium cholate and either 2.5 μCi 14C cholate or 12.5 μCi 3H cholate per 10 ml.
SERUM PROGESTERONE
Ten millilitres of blood were obtained by venepuncture with each bile sample. The serum was separated and snap frozen at −70°C for subsequent batch hormone analysis. The serum progesterone was determined by radioimmunoassay after hexane extraction.

STATISTICS
A precise measure of the cholesterol saturation of duodenal bile requires multiple samples. Therefore, the average of the two or three cholesterol saturation index measurements in each phase of the study was used for statistical analysis. The Wilcoxon’s signed rank test for paired samples was used to analyse all data.

Results
Seven of the subjects completed all phases of the study, while the other three were able to participate only in a control phase and two oestrogen/progestagen phases because they left the area. The control menstrual cycles proved to be ovular in all subjects by measurement of serum progesterone levels. The oestrogen/progestagen combinations induced an anovular cycle in all subjects except one. This subject ovulated while taking 30 μg ethinylestradiol alone. The daily dose of synthetic female sex hormone(s) in each phase was tolerated well by all women except one who developed early morning nausea and vomiting while taking 30 μg ethinylestradiol plus 150 μg norgestrel. None of the women had major changes in their weight during the year of study.

CHOLESTEROL SATURATION OF GALL-BLADDER BILE
The average cholesterol saturation index of gall-bladder bile in each subject’s control menstrual cycle was compared with that in each oestrogen/progestagen phase of the study (Fig. 1). Variation between the multiple cholesterol saturation index values obtained for each subject in each phase of the study was less than we found in a previous study, the mean range of values being 0.17±0.04 (SEM). No significant change in the cholesterol saturation index occurred when the subjects ingested a daily dose of 30 μg ethinylestradiol alone or 30 μg ethinylestradiol plus 2.5 mg norethisterone. A significant increase in the median cholesterol saturation index was observed, however, when the subjects ingested a daily dose of both 30 μg ethinylestradiol plus 150 μg norgestrel (p=0.01), and 50 μg ethinylestradiol plus 250 μg norgestrel (p<0.01). Another important observation was that while only one of the 10 subjects had bile supersaturated in cholesterol during her normal menstrual cycle, this increased to five of the 10 subjects while taking 30 μg ethinylestradiol plus 150 μg norgestrel.

Comparisons of the subject’s gall-bladder bile cholesterol saturation index in each of the oestrogen/progestagen phases of the study are shown in Fig. 2. A significant increase in the median cholesterol saturation index was observed when 150 μg norgestrel was added to 30 μg ethinylestradiol (p<0.02). An increase in the cholesterol saturation index was also observed between the 30 μg ethinylestradiol alone and the 50 μg ethinylestradiol plus 250 μg norgestrel but it did not quite reach significance, possibly because of the smaller sample size. No significant difference in the cholesterol saturation index was found between 30 μg ethinylestradiol plus 150 μg norgestrel and 50 μg ethinylestradiol plus 250 μg norgestrel, nor 30 μg ethinyl-
The effect of synthetic female sex hormones on cholesterol saturation index of gall-bladder bile in normal women. Mean of two bile CSI measurements taken during the last week of second month of ingestion of each synthetic female sex hormone combination have been compared in each subject. EE: ethinylestradiol, Nor: norethisterone, Ng: norgestrel.

Epidemiological studies have shown that the use of oral contraceptives has changed the prevalence and incidence of gall-stone disease. More recent biochemical studies have shown that some oral contraceptives increase the cholesterol saturation index of bile and thus its lithogenic potential. These studies had limitations. Initially, up to five commercial brands of oral contraceptives were used, and these contained several different synthetic female sex hormones (50 μg ethinylestradiol plus 500 μg norgestrel, 50 μg mestranol plus 1·0 mg norethindrone, 50 μg ethinylestradiol plus 1·0 mg ethyndiol diacetate, and 0·1 mg mestranol plus 1·0 mg ethyndiol diacetate). It was, therefore, impossible to determine whether the observed change in the cholesterol saturation index was an oestrogenic or progestagenic effect. Secondly, these 'high dose' oestrogen contraceptives are now prescribed only where lower dose oestrogen contraceptives prove inadequate. It is possible that the

Fig. 2 Comparison of effect of ingestion of different synthetic female sex hormone combinations on cholesterol saturation index of gall-bladder bile in normal women. The effect of synthetic female sex hormone combinations on the bile acid pool size is shown in Fig. 3. None of the synthetic female sex hormones had a significant effect on the size of the total bile acid pool. There was no significant change in the size of the total bile acid pool during the last week of the second month of synthetic female sex hormone ingestion. EE: ethinylestradiol, Nor: norethisterone, Ng: norgestrel.
effects which have been observed with high dose oestrogen oral contraceptives do not occur with the lower dose preparations, especially as most studies\textsuperscript{17-21} have shown that the physiological changes in natural female sex hormones during a normal menstrual cycle do not alter the cholesterol saturation index of bile.

The daily ingestion of 30 $\mu$g ethinylestradiol alone in this study had no significant effect on the degree of cholesterol saturation in bile even though the cycles were anovular. This suggests that the increase in the biliary cholesterol saturation index which was observed with ethinylestradiol plus norgestrel cannot be attributed to ethinylestradiol, nor the suppression of physiological oestrogens, progesterone, luteinising hormone, or follicular stimulating hormone. Only one other study on the effect of 30 $\mu$g ethinylestradiol has been carried out, and it was performed in men, not women.\textsuperscript{37} It showed that this dose of ethinylestradiol resulted in a significant rise in the biliary cholesterol saturation index after seven days' treatment. The reason for this sex difference is unclear, but it may be related to the 50% fall in the levels of circulating testosterone which also accompanied the use of ethinylestradiol in men.\textsuperscript{37}

The importance of the increase in the biliary cholesterol saturation index which was observed with the combined oral contraceptives containing 30 $\mu$g ethinylestradiol plus 150 $\mu$g norgestrel, and 50 $\mu$g ethinylestradiol plus 250 $\mu$g norgestrel, is highlighted by the fact that the number of women with bile supersaturated in cholesterol increased from 10% during a normal menstrual cycle to 50% while on therapy. Furthermore, the addition of 150 $\mu$g norgestrel to 30 $\mu$g ethinylestradiol also resulted in a significant increase in the biliary cholesterol saturation index. It is surprising, therefore, that the addition of a pharmacologically equivalent dose of another progestagen, norethisterone, to 30 $\mu$g ethinylestradiol did not have a similar effect. These findings indicate that norgestrel, and not ethinylestradiol, was responsible for increasing the degree of cholesterol saturation in bile. The theory\textsuperscript{26-28} that the lithogenic effect of oral contraceptives on bile is solely because of oestrogens is, therefore, no longer tenable. It is still possible, however, that higher doses of ethinylestradiol or other synthetic oestrogens – for example, mestranol – might affect the biliary cholesterol saturation index, or that oestrogens are necessary for norgestrel to exert its effect. The latter possibility might have been answered if side effects had not prevented subjects from participating in a 150 $\mu$g norgestrel alone phase of this study.

The reason why norgestrel affected the cholesterol saturation index of bile and norethisterone did not, could be related to the differing properties of synthetic progestagens. It can be calculated from the figures of Dickey and Stone\textsuperscript{38} that, while 150 $\mu$g norgestrel and 2.5 mg norethisterone are approximately equivalent doses of progestagen, norgestrel is four to five times more androgenic and seven times more anti-oestrogenic than norethisterone. Furthermore, norethisterone has a mild oestrogenic effect which norgestrel lacks. As norgestrel is a potent anti-oestrogen and yet androgenic, an oral contraceptive which contained ethinylestradiol 30 $\mu$g plus 150 $\mu$g norgestrel would have a lower oestrogen to progestagen potency ratio than ethinylestradiol 30 $\mu$g plus 2.5 mg norethisterone.\textsuperscript{39} The overall oestrogen to progestagen potency ratio of an oral contraceptive may, therefore, be more important in determining its effects on the biliary cholesterol saturation index than the dose of each sex hormone. This theory already has some support as an inverse correlation has been shown between the oestrogen to progestagen ratio of oral contraceptives and the risk of developing gall stones.\textsuperscript{6} Future studies into the effect of sex hormones on bile should consider not only the doses of oestrogen and progestagen, but also the overall oestrogen to progestagen potency ratio.

None of the oestrogen/progestagen combinations used in our study significantly changed the total bile acid pool size or the bile acid profile of bile, except for ethinylestradiol alone, which decreased the proportion of chenodeoxycholic acid. Other studies\textsuperscript{19 22 23} have also found that both endogenous and exogenous female sex hormones are associated with a decreased proportion of chenodeoxycholic acid in bile. It is not clear, however, whether this change is related to an increase in the cholesterol saturation index of bile. Our results also show that increases in the biliary cholesterol saturation index can occur with combination oral contraceptives without concomitant changes in bile acid composition, and support the more recent study of Bennion et al.\textsuperscript{40} They were unable to show a change in total bile acid pool size or bile acid composition with oral contraceptives, even though the biliary cholesterol saturation index increased. Instead, they showed that the increase in the biliary cholesterol saturation index was because of hepatic enhancement of cholesterol secretion into bile.

In conclusion, our study has shown that the progestagen, norgestrel, and not the oestrogen, ethinylestradiol, is responsible for the increase in bile cholesterol saturation which accompanies the use of oral contraceptives.\textsuperscript{23-25} It also suggests that this side effect is not a property of all progestagens.
If future studies confirm these findings, it may be possible to manufacture oral contraceptives which do not subject women to an increased risk of gallstone formation.

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