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EFFECT OF ESTROGEN–PROGESTIN COMBINATIONS ON THE HEPATIC MICROSOMAL METABOLISM OF MESTRANOL

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Abstract—Three combinations of steroid contraceptive drugs (mestranol plus lynestrenol, norethindrone or norethynodrel) were given orally at effective antifertility doses for 30 days to rats, mice and guinea pigs. Eighteen hr after the last treatment, the animals were sacrificed for preparation of liver microsomal enzymes and the evaluation of mestranol metabolism *in vitro*.

The results obtained indicate that these three animal species convert mestranol into ethynylestradiol, a hormonally active agent, which is further metabolized into more polar metabolites.

A prior administration of contraceptive agents increases in rats and mice the disappearance of mestranol and the metabolism of ethynylestradiol *in vitro*. In guinea pigs the effect was much less marked.

The results are discussed considering that the estrogen activity of mestranol *in vivo* depends on the availability of ethynylestradiol for the estrogen receptors.

Mestranol (17-α-ethynyl-3-methoxy-1,3,5(10)-estratrien-17-β-ol) is widely prescribed as an orally administered estrogen compound, particularly used in contraceptive formulations.

Several reports indicate that mestranol acts as an estrogen only after the formation of ethynylestradiol, a hormonally active compound formed by the liver microsomal *O*-demethylase, which can readily interact with the binding sites of estrogen receptor protein in the target tissues [1, 2]. Thus, the therapeutic activity of mestranol in the organism depends on its biotransformation to ethynyl-estradiol and on the availability of this latter compound for the estrogen receptors.

Individual differences in the metabolism of mestranol may lead, therefore, to an alteration of the estrogenic effectiveness of mestranol [2, 3]. In addition some progestational compounds added *in vitro* have been shown to inhibit the *O*-demethylation of mestranol [2].

The aim of this work was to investigate the effect of some of the most widely used steroid contraceptive drug (SCD) combinations on mestranol metabolism in experimental conditions duplicating human clinical utilization. Therefore the SCD combinations were administered chronically to three animal species for periods covering more than one estrus cycle, in doses which produced an experimentally controlled antifertility effect. At the end of the treatment *O*-demethylation of mestranol by liver microsomes *in vitro* was measured by determining the disappearance of mestranol and the presence of ethynylestradiol.

MATERIALS AND METHODS

Animals. Female Charles River rats (body wt 220 ± 10 g), female CD_1 mice (body wt 25 ± 3 g) and female PIR bright/Z guinea pigs (body wt 350 ± 30 g) were used in all experiments.

The rats and mice were housed in makrolon cages (six per cage) and the guinea pigs in steel rod cages (five per cage). The animals were kept at a constant room temperature of 22° with a relative humidity of 60%. Food and water were given *ad lib.*

Drug administration. The following SCD combinations were used in rats and mice: lynestrenol (5 mg/kg) plus mestranol (0.3 mg/kg); norethindrone (4 mg/kg) plus mestranol (0.2 mg/kg); norethynodrel (4 mg/kg) plus mestranol (0.06 mg/kg). In guinea pigs the same combinations were used at the following doses: lynestrenol (1.25 mg/kg) plus mestranol (0.075 mg/kg), norethindrone (4 mg/kg) plus mestranol (0.2 mg/kg); norethynodrel (1 mg/kg) plus mestranol (0.015 mg/kg).

The dose of each drug combination was selected according to the previously established antifertility effect [5] as measured by a bioassay procedure, developed by Kincl and Dorfman [4] in the different animal species (ED_{95}).

The drugs were dissolved in corn oil and given orally for a period of 30 days to rats and mice and 32 days to guinea pigs which in this animal species is the length of two estrus cycles. Controls received only corn oil. Animals were sacrificed by decapitation 18 hr (at 10:00 a.m.) after the last treatment. Repeated analyses of mestranol and ethynylestradiol in several liver microsomal preparations, indicated that no traces of steroid contraceptives are present in liver microsomal preparations of the three animal species tested.

Preparation of liver microsomes. Following animal sacrifice the livers were immediately removed and homogenized in ice-cold 1.15% KCl solution (1:4 w/v) with a Teflon–glass homogenizer. The homogenate was centrifuged at 9000 *g* for 20 min and the supernatant fraction was centrifuged again at 105,000 *g* for 1 hr (rotor 40'-Beckman Model L ultracentrifuge).

Incubation in vitro. Microsomal suspension in 1.15% KCl solution containing 8-10 mg protein/ml were used. Protein determinations were carried out according to Lowry's method [6]. Each incubation mixture consisted of 2.5 ml of microsomal suspension

Table 1. Disappearance of mestranol (M) and formation of ethynylestradiol (EE) with liver microsomes obtained from different animal species after 30 days of treatment with lynestrenol + mestranol

Animal species	Exptl. group	Parameter measured	Concentrations of mestranol (μg/5 ml) 100	300	500
Rat	Controls	% M unchanged	39 ± 3	65 ± 5	73 ± 3
		% EE found	33 ± 1	16 ± 2	9 ± 1
	Treated*	% M unchanged	8 ± 3	47 ± 5	66 ± 3
		% EE found	22 ± 2	24 ± 4	14 ± 2
Mouse	Controls	% M unchanged	18 ± 1	56 ± 4	78 ± 5
		% EE found	21 ± 3	27 ± 3	21 ± 2
	Treated*	% M unchanged	9 ± 1	15 ± 2	52 ± 3
		% EE found	<0.6	38 ± 3	38 ± 5
Guinea pig	Controls	% M unchanged	44 ± 4	75 ± 4	89 ± 4
		% EE found	27 ± 2	63 ± 3	75 ± 1
	Treated*	% M unchanged	32 ± 2	18 ± 3	11 ± 1
		% EE found	40 ± 2	33 ± 1	22 ± 2

Thirty-day treatment with lynestrenol + mestranol.
* 5 ± 0.3 mg/kg orally.
† 1.25 ± 0.075 mg/kg orally.
Value are mean ± S.E.M. of at least 4 determinations—30 min incubation.

equivalent to 1 g of liver; NADP (1.5 μmoles); glucose-6-phosphate (50 μmoles); glucose-6-phosphate dehydrogenase (0.5 units); magnesium chloride (25 μmoles), nicotinamide (50 μmoles); 1.4 ml of 0.2 M phosphate buffer pH 7.4; mestranol (100–500 μg); 0.45 ml of 1.15% KCl and water to obtain a final volume of 5 ml. The concentrations of mestranol or ethynylestradiol were selected after preliminary experiments. The mixtures were incubated in a Dubnoff metabolic shaker at 37 under air for various periods of time. At the end of the incubation period the mixture was extracted twice with 10 ml of ether–chloroform (3:1 v/v). The combined organic extracts were evaporated to dryness, redissolved in an acetone solution of the internal standard, and then gaschromatographed.

Chemical determination. The analyses of mestranol and its metabolite ethynylestradiol (EE) were carried out by using a gas chromatograph (Model GI, Carlo Erba, Milan) equipped with a flame ionization detector. The stationary phase was OV 17, 3% on Gas Chrom Q (100–120 mesh) packed into a 2-m glass column (3 mm i.d., 6 mm o.d.). The flow rate of carrier gas (nitrogen) was 30 ml/min and the column temperature was 265 .

For the quantitative steroid analysis the internal standard technique was used. 2,4-Dinitrophenylhydrazone of camphor was chosen as an internal standard, because of its suitable retention time.

Recovery studies of mestranol and its main metabolite, 17-α-ethynyl-estradiol, from microsomes incubation mixtures of the three animal species were satisfactory ranging from 75 ± 2% to 90 ± 2%.

RESULTS

Effect of a 30-day treatment with lynestrenol plus mestranol (L + M). The combination of L + M given 30 days to rats and mice (5 + 0.3 mg/kg orally) or to guinea pigs (1.25 + 0.075 mg/kg orally) is capable of influencing the rate of metabolism of mestranol *in vitro* (*O*-demethylation) by liver microsomal enzymes.

The results obtained are summarized in Table 1 (different concentrations of M added *in vitro*) and in Table 2 (different times of incubation). For the three animal species it is evident that M disappears at a faster rate from liver microsomes obtained from animals pretreated with L + M with respect to controls.

Although the data do not allow strict comparison it seems possible to suggest that the increased disappearance of mestranol in animals pretreated with L + M is more marked in rats and mice than in guinea pigs.

The formation of EE cannot be entirely calculated because this hormonally active metabolite is further metabolized to unknown compounds in all three animal species. Data reported in Table 7 (controls) indicates in fact that EE disappears from liver microsomes *in vitro* at an increasing rate going from guinea pigs to rats and to mice.

In any case it is evident that the amount of EE can be found at the end of incubation with M is decreased for rats and mice while it is increased for guinea pigs.

Effect of a 30-day treatment with norethindrone + mestranol (Ne + M). The combination Ne + M (4 + 0.2 mg/kg orally) given for 30 days to rats, mice and guinea pigs increases the disappearance of mestranol *in vitro* by liver microsomal enzymes only for rats and mice but not for guinea pigs.

Tables 3 and 4 summarize these findings by using different concentrations of M and different times of incubation with a given M concentration. As far as the presence of EE at the end of the incubation, there is a marked decrease in mice while for rats and guinea pigs there is not a significant change when the animals are pretreated with the combination Ne + M with respect to controls.

Effect of a 30-day treatment with norethynodrel + mestranol (Nl + M). The results obtained with the combination Nl + M given for 30 days to rats and mice (4 + 0.06 mg/kg orally) or to guinea pigs

Table 2. Disappearance of mestranol (M) and formation of ethynylestradiol (EE) with liver microsomes obtained from different animal species after 30 days of treatment with lynestrenol + mestranol

Animal species	Exptl. group	Parameter measured	Min after incubation with mestranol (100 μg/5ml) 10	30	60	120
Rat	Controls	% M unchanged	53 ± 3‡	39 ± 3	28 ± 2	23 ± 1
		% EE found	30 ± 2	33 ± 1	33 ± 2	32 ± 3
	Treated	% M unchanged	26 ± 1	8 ± 3	9 ± 2	8 ± 2
		% EE found	33 ± 2	22 ± 2	22 ± 3	21 ± 1
Mouse	Controls	% M unchanged	51 ± 4	18 ± 1	9 ± 2	6 ± 1
		% EE found	31 ± 3	21 ± 3	17 ± 2	6 ± 1
	Treated*	% M unchanged	22 ± 2	9 ± 1	6 ± 1	6 ± 1
		% EE found	38 ± 3	<0.6	<0.6	<0.6
Guinea pig	Controls	% M unchanged	74 ± 3	44 ± 4	40 ± 1	31 ± 2
		% EE found	25 ± 3	32 ± 2	32 ± 3	32 ± 2
	Treated†	% M unchanged	63 ± 2	27 ± 2	16 ± 3	6 ± 1
		% EE found	35 ± 2	40 ± 2	48 ± 3	48 ± 3

Thirty-day treatment with lynestrenol + mestranol.
* 5 ± 0.03 mg/kg orally.
† 1.25 ± 0.045 mg/kg orally (n = 4).
‡ Values are mean ± S.E.M.

(1 + 0.015 mg/kg orally) are presented in Tables 5 and 6. It is evident that liver microsomes obtained from rats and mice pretreated with Nl + M metabolize M at a faster rate than untreated animals. On the contrary the liver microsomes of guinea pigs pretreated with Nl + M are less effective than controls in metabolizing M particularly when different times of incubation are considered (see Table 6).

The presence of EE at the end of the incubation is also differently affected by the treatment with Nl + M in the three animal species. In fact, while with liver microsomes from mice there is a marked decrease, with liver microsomes of guinea pigs there is a slight decrease and with liver microsomes from rats a moderate increase.

However, while the decrease of EE in mice is accompanied by an increased metabolism of M, the decreased level of EE in guinea pigs is related to a reduced disappearance of M.

Effect of treatment with mestranol on ethynylestradiol metabolism. Data reported in Table 7 show that EE can be metabolized by liver microsomal enzymes. The rate of disappearance of EE is faster with liver microsomes of mice followed in decreasing order by liver microsomes of rats and guinea pigs.

A previous treatment with M (6 mg/kg *per os* for 3 days) increases the disappearance of EE by liver microsomes of rats and mice while it decreases the disappearance of EE in guinea pigs after a 3 day treatment at the dose of 3 mg/kg twice a day.

DISCUSSION

The reported results indicate first that liver microsomal enzymes are capable of metabolizing mestranol with the formation, through an *O*-demethylation, of the hormonally active agent ethynylestradiol. This metabolizing activity occurs at the highest rate with

Table 3. Disappearance of mestranol (M) and formation of ethynylestradiol (EE) with liver microsomes obtained from different animal species after 30 days of treatment with norethindrone + mestranol (4 + 0.2 mg/kg oral)

Animal species	Exptl. group	Parameter measured	Concentrations of mestranol (μg/5 ml) 100	300	500
Rat	Controls	% M unchanged	39 ± 1*	65 ± 5	73 ± 4
		% EE found	33 ± 3	16 ± 1	9 ± 2
	Treated*	% M unchanged	12 ± 1	54 ± 3	65 ± 3
		% EE found	37 ± 4	27 ± 2	14 ± 1
Mouse	Controls	% M unchanged	18 ± 1	56 ± 4	78 ± 5
		% EE found	21 ± 3	27 ± 3	21 ± 2
	Treated	% M unchanged	11 ± 1	25 ± 2	50 ± 3
		% EE found	<0.6	33 ± 5	36 ± 3
Guinea pig	Controls	% M unchanged	44 ± 4	78 ± 4	79 ± 4
		% EE found	32 ± 2	18 ± 3	11 ± 1
	Treated	% M unchanged	58 ± 4	74 ± 4	84 ± 5
		% EE found	27 ± 3	16 ± 1	10 ± 1

Thirty min of incubation
* Values are mean ± S.E.M. (n = 4).

Table 4. Disappearance of mestranol (M) and formation of ethynylestradiol (EE) with liver microsomes obtained from different animal species after 30 days of treatment with norethindrone + mestranol (4 mg + 0.2 mg/kg oral)

Animal species	Exptl. group	Parameter measured	Min after incubation with mestranol (100 μg/5 ml) 10	30	60	120
Rat	Controls	% M unchanged	53 ± 2*	39 ± 1	28 ± 2	23 ± 1
		% EE found	29 ± 2	33 ± 3	33 ± 2	32 ± 2
	Treated	% M unchanged	27 ± 1	12 ± 1	11 ± 0.5	11 ± 1
		% EE found	38 ± 3	37 ± 4	37 ± 2	26 ± 3
Mouse	Controls	% M unchanged	51 ± 4	18 ± 1	9 ± 2	6 ± 1
		% EE found	31 ± 3	21 ± 3	17 ± 2	6 ± 1
	Treated	% M unchanged	26 ± 3	11 ± 1	7 ± 1	6 ± 1
		% EE found	33 ± 2	<0.6	<0.6	<0.6
Guinea pig	Controls	% M unchanged	74 ± 3	44 ± 4	40 ± 1	31 ± 2
		% EE found	25 ± 3	32 ± 2	32 ± 3	32 ± 2
	Treated	% M unchanged	74 ± 2	58 ± 4	49 ± 4	32 ± 2
		% EE found	14 ± 3	27 ± 3	26 ± 2	28 ± 1

* Values are mean ± S.E.M. (n = 4).

the liver microsomes of mice followed in decreasing order by liver microsomes of rats and guinea pigs. Furthermore, ethynylestradiol can be further metabolized by liver microsomes at a rate which is decreasing from mice to rats and to guinea pigs.

These findings are in agreement with other investigations carried out *in vivo* [2] and with isolated perfused rat liver [7] showing that mestranol is *O*-demethylated and that the resulting ethynylestradiol is transformed into 2-hydroxyethynylestradiol and other, yet unidentified, more polar metabolites.

Since mestranol is utilized in several contraceptive combinations for women, our experiments to investigate whether prolonged contraceptive treatments were able to alter the rate of mestranol metabolism by liver microsomal enzymes were particularly interesting. The three SCD combinations were given at different doses to the three animal species over a prolonged period. The results obtained clearly indicate that the animal species is an important variable. In rats and mice, but not in guinea pigs, there is on the whole, following SCD treatment an increased *in vitro* disappearance of mestranol and a relatively lower level of ethynylestradiol with respect to controls.

The kinetics of mestranol disappearance and ethynylestradiol formation are such as to establish that the ethynylestradiol measured is the net result of the amount formed with respect to the amount metabolized.

In this respect it may be tentatively concluded that SCD treatments increase in rats and mice both the transformation of mestranol into ethynylestradiol and the further metabolization of ethynylestradiol.

However, the type of contraceptive combination may be also of importance in determining changes of mestranol by liver microsomes.

In fact, in rats the presence of ethynylestradiol after incubation with liver microsomal enzymes is decreased with respect to controls when the animals

Table 5. Disappearance of mestranol (M) and formation of ethynylestradiol (EE) with liver microsomes obtained from different animal species after 30 days treatment with norethynodrel and mestranol

Animal species	Exptl. group	Parameter measured	Concentrations of mestranol (μg/5 ml) 100	300	500
Rat	Controls	% M unchanged	39 ± 1‡	65 ± 5	73 ± 4
		% EE found	33 ± 3	16 ± 1	9 ± 2
	Treated*	% M unchanged	14 ± 2	47 ± 3	67 ± 5
		% EE found	38 ± 4	28 ± 3	17 ± 3
Mouse	Controls	% M unchanged	18 ± 4	56 ± 4	78 ± 5
		% EE found	21 ± 3	27 ± 3	21 ± 2
	Treated*	% M unchanged	8 ± 2	28 ± 3	59 ± 4
		% EE found	2 ± 0.5	39 ± 3	34 ± 4
Guinea pig	Controls	% M unchanged	45 ± 2	82 ± 4	85 ± 5
		% EE found	34 ± 3	16 ± 3	11 ± 1
	Treated†	% M unchanged	60 ± 4	82 ± 4	85 ± 5
		% EE found	25 ± 3	14 ± 3	9 ± 1

Thirty-day treatment with norethynodrel + mestranol. Thirty-min incubation.
* 4 ± 0.06 mg/kg orally.
† 1 ± 0.015 mg/kg orally.
‡ Values are mean ± S.E.M. (n = 4).

Table 6. Disappearance of mestranol (M) and formation of ethynylestradiol (EE) with liver microsomes obtained from different animal species after 30 days of treatment with norethynodrel + mestranol

Animal species	Exptl. group	Parameter measured	Min after incubation with mestranol (100 μg/5 ml) 10	30	60	120
Rat	Controls	% M unchanged	53 ± 2‡	39 ± 1	28 ± 2	23 ± 1
		% EE found	29 ± 2	33 ± 3	33 ± 2	32 ± 3
	Treated*	% M unchanged	24 ± 3	14 ± 2	13 ± 2	11 ± 1
		% EE found	42 ± 3	38 ± 4	34 ± 3	29 ± 2
Mouse	Controls	% M unchanged	51 ± 4	18 ± 1	9 ± 2	6 ± 1
		% EE found	31 ± 3	21 ± 3	17 ± 2	6 ± 1
	Treated*	% M unchanged	26 ± 3	8 ± 2	8 ± 1	7 ± 2
		% EE found	35 ± 2	2 ± 0.5	<0.6	<0.6
Guinea pig	Controls	% M unchanged	74 ± 3	45 ± 2	42 ± 3	33 ± 2
		% EE found	19 ± 1	34 ± 3	33 ± 2	35 ± 4
	Treated†	% M unchanged	70 ± 2	60 ± 4	58 ± 1	57 ± 2
		% EE found	11 ± 1	25 ± 3	26 ± 2	28 ± 4

Thirty-day treatment with norethynodrel + mestranol.
* 4 + 0.06 mg/kg orally.
† 1 + 0.015 mg/kg orally.
‡ Values are mean ± S.E.M. (n = 4).

were pretreated with lynestrenol + mestranol, is increased with norethynodrel + mestranol and is unchanged with norethindrone + mestranol.

In the guinea pig, although the changes are relatively small, the pretreatment with lynestrenol + mestranol increases the disappearance of mestranol and the presence of ethynylestradiol, while the combination norethynodrel + mestranol gives the opposite effect and the treatment with norethindrone + mestranol has no effect.

The effects observed with the SCD treatments do not appear to depend on the dose of mestranol because in mice the same increase of mestranol metabolism was observed with doses of the estrogen component ranging from 0.06 to 0.3 mg/kg *per os*.

On the other hand, no relationship was observed with doses ranging from 0.015 to 0.2 mg/kg *per os* in guinea pigs. Although *ad hoc* experiments must be performed it appears likely that the individual progestin component may have a marked influence depending on the species and on the metabolism of mestranol and ethynylestradiol.

In this study the dosage of SCD preparation was that found to be the minimal effective antifertility doses in the animal species.

The significance of this investigation is that the estrogen component may change during the treatment particularly when the administration is prolonged.

This may be especially important with estrogen compounds such as mestranol which do not bind, *per se*, to estrogen receptors [1, 2], but require a previous biotransformation.

Owing to species variability it is impossible to attempt any extrapolation from these studies to human use of contraceptive medication. However, the present findings strongly suggest the need of similar investigations in women to ascertain whether a change in the availability of the estrogen component occurs in the prolonged administration of contraceptive treatment.

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Table 7. Disappearance of ethynylestradiol (EE) from liver microsomes obtained from different animal species after 3 days of oral treatment with mestranol twice a day (6 mg/kg) in rats and in mice and 3 mg/kg in guinea pigs

Animal species	Exptl. group	Min after incubation with ethynylestradiol (100 μg/5 ml) 10	30	60	120
Rat	Controls*	45 ± 1	37 ± 1	34 ± 2	34 ± 2
	Treated	17 ± 1	8 ± 1	5 ± 1	5 ± 1
Mouse	Controls	45 ± 3	18 ± 2	5 ± 1	2 ± 1
	Treated	40 ± 2	9 ± 1	5 ± 1	2 ± 1
Guinea pig	Controls	70 ± 3	60 ± 4	50 ± 2	45 ± 1
	Treated	84 ± 2	71 ± 3	62 ± 4	58 ± 3

Figures represent the % of EE found in respect to the added concentration (n = 4).
* Values are means ± S.E.M.

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