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Teratogenicity and Embryotoxicity of Orally Administered Lynestrenol in Rabbits

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Abstract. Pregnant Belted Dutch rabbits were administered lynestrenol 17-α-ethynyl-oestr-4-en-17-β-ol) orally on days 6–18 of gestation at doses of 0.1, 0.5, and 2.5 mg/kg/day. On day 29 of gestation the does were killed and autopsied and the fetuses were examined for external, visceral and skeletal abnormalities.

Lynestrenol administration produced a statistically significant increase in the number of post-implantation loss ($p = 0.05$) and in the average per cent of abnormal fetuses per dose group (63%, 66%, and 87% for the medicated group, versus 12% for the placebo group, $p = 0.05$).

None of the doses tested was lethal to the does, but the average weight gain was decreased for the medium and the high dose groups.

Abnormalities of the central nervous system and skeletal variants were the most frequent findings in the fetuses.

Key words: Lynestrenol – Progestogen – Teratogenesis – Embryotoxicity – Rabbits

Introduction

Lynestrenol is a potent oral progestogen used as a hormonal contraceptive. Exposure to fetuses during early pregnancy may occur by inadvertent initiation or continuation of hormonal contraception during pregnancy. The widespread use of hormonal contraceptives involves a potential risk for the progeny and thus has been a matter of concern to many health authorities. A WHO scientific group on the effect of female sex hormones on fetal development and infant health (WHO 1981) analyzed the aspects of exposure of fetuses or newborn infants to female sex hormones as classified into exposure prior to conception, exposure during pregnancy and exposure through breast milk. On reviewing different epidemiological studies of birth defects and exposure to oral

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contraceptives during pregnancy, the scientific group concluded that the evidence for an increased risk of congenital malformations is not clear, and, that if such risks exist, they are very small.

As for hormonal pregnancy tests and supportive hormone therapy, the group concluded that the possibility of these uses constituting a small increased risk of congenital malformations cannot be ruled out.

As far as individual hormones are concerned, the group noted that there has been little research into possible effects on the fetus of mini-pills containing progestogen only.

Lynestrenol, which is used as a contraceptive mini-pill, was studied with whole-body autoradiography and liquid scintillation counting following the oral administration of radiolabelled compound to pregnant rats (Sannes and Nafstad 1980). In this study, transplacental distribution of lynestrenol was observed. To our knowledge, no animal experiment to examine a potential teratogenic effect has been carried out. Hence, a study to examine the possible effects of lynestrenol upon the fetal development in rabbits was undertaken.

Materials and Methods

Test Material

Lynestrenol (17 α-ethynyl-oestr-4-en-17-β-ol), a synthetic progestogen, was used as Exlutona tablets produced by Organon, Oss, The Netherlands.

Animals and Housing

Nulliparous female Belted Dutch rabbits of a local non-inbred strain were used. The animals were 16–18 weeks of age, weighed 1.6–3.0 kg and were of a conventional hygienic quality. Prior to the experiment they were tested serologically with the india ink immunoreaction test for Encephalitozoon cuniculi-infection (Waller et al. 1979) and found negative. The bucks used for semen donation were Belted Dutch rabbits at 4–12 months of age of the same local strain as the does.

The animals were housed separately in metal cages measuring 45 × 45 × 38 cm equipped with automatic watering system. They were fed ad libitum with a commercial pelleted breeding diet for rabbits and guinea pigs (Ewos, Södertälje, Sweden). Room temperature was 20–22° C, relative humidity was 40–60%, and there was a 12 : 12 h lighting cycle.

Experimental Design

Experiment 1. Artificial insemination was performed according to Lenz (1976) with the modification described by Lyngset and Sannes (1979). The day of insemination was designated as day 0 of gestation. Groups of 14–20 females were administered orally with 0, 0.1, 0.5, and 2.5 mg lynestrenol per kg body weight from day 6 to day 18 of gestation. The tablets were administered with an applicator (Lyngset and Sannes 1979); the control group was administered placebo tablets containing the vehicle only with the same application procedure.

Each animal was weighed weekly on the same week day. The experiment was performed during March to June.

A preliminary study had been performed to evaluate the suitability of the rabbit strain for teratology testing. The model was found to respond with fetal malformations after a challenge with a high dose of vitamin A and to the administration of 0.5 mg/kg lynestrenol, respectively (unpublished results).

Experiment 2. The experiment was performed with the same design as in Experiment 1, with the exception that each group consisted of five to six animals, that it was performed during the months August to October and that the highest dose level (2.5 mg/kg) was omitted.

Maternal and Fetal Observations

In Experiment 1, the does were killed by a blow on the head on day 29 of gestation and subjected to autopsy. The uteri were examined, the number of implantation sites and live and dead fetuses were registered.

The live fetuses were sacrificed by an i.p. injection of 0.5 ml pentobarbitone (10%). All fetuses were weighed and sexed. Approximately two-thirds of the fetuses from each litter were fixed in Bouin's solution and examined for soft tissue alterations with the razor blade technique of Wilson (1965) slightly modified. The modification consisted of gross inspection of the viscera instead of sectioning the body. The remaining fetuses were examined for skeletal abnormalities according to the alizarin staining method of Dawson (1926). The viscera from all fetuses were examined.

In Experiment 2, the offspring was kept alive for 4 weeks in order to examine the post-natal development. Daily observations of behaviour and general health condition were made. After 4 weeks the weanlings were anaesthetized with thiopentone, exsanguinated by heart puncture and subjected to autopsy.

Samples from selected material from both experiments were fixed in 10% buffered formalin and prepared for histological examination of sections stained with hematoxylin and eosin.

Statistical Evaluation

The litter was considered the experimental unit for analysis of data regarding embryotoxicity and teratogenicity. Average percentages were calculated for each examination technique according to the formula: $100 \times \Sigma$ [No. of malformed fetuses in the litter/total number of fetuses examined in the litter]/total number of litters, as described by Marks et al. (1981).

Pairwise comparisons of group means to the vehicle control group mean were calculated by the Fisher-Irwin significance test (Hodges and Lehmann 1970).

Results

Most of the animals in the highest dose groups and some in the medium dose groups of both experiments revealed temporary anorexia from 5 to 7 days after the medication was started. In some cases traces of blood and/or remnants of fetuses were found in the cages indicating that these animals had aborted.

Experiment 1. The mean body weight gain of pregnant rabbits in the two highest dose groups was reduced during the gestation period as compared to the controls. The average weight gain from day 6 to day 29 was +163 g for the control group, +139 g for the low dose group, +63 g for the medium dose group, and +100 g for the high dose group (Table 1).

Table 1. Effect of lynestrenol on reproduction in rabbits[a]

	Control[b]	Dose (mg/kg/day)		
		0.1	0.5	2.5
Number of pregnant does examined	14	14	16	20
Number of litters with live fetuses	14	14	15	2
Average weight gain (g) during pregnancy (days 6–29)	163 ± 17	139 ± 33	63 ± 41	100 ± 35
Average number of implantation sites[c]	5.5	6.5	6.6	6.1
Average number of live fetuses pr. litter	5.4	5.6	5.8	5.5
Post implantation loss (%)[d]	1.3	13.2*	17.1*	90.9*
Average fetal weight (g)[e]	34.8 ± 0.9	37.6 ± 0.7	37.1 ± 0.6	35.0 ± 1.2
Female/male live fetuses	37/39	44/35	43/44	2/5[f]

[a] Killed on day 29 of gestation after receiving lynestrenol (p.o.) on days 6–18
[b] Received vehicle on days 6–18
[c] Per pregnant female
[d] Includes resorptions, abortions and dead fetuses
[e] Dead and aborted fetuses were excluded
[f] The sex of four fetuses was not determined
* Statistically significant higher than the control group

No significant lesions were observed on post mortem examination of the does.

There was no difference in the average number of implantation sites between experimental and control groups. However, the post-implantation loss increased significantly with increasing dose levels. The reproduction data are presented in Table 1.

The results of the teratological examination are presented in Table 2. Among litters with live fetuses in the medicated groups, the incidence of malformations was significantly increased over the control values.

Analysis of different categories of malformations in the medicated groups showed that the average percentages of CNS/eye, skeletal, and cardiovascular abnormalities were significantly increased over the control values.

The types of CNS/eye abnormalities included varying degrees of dilated cerebral ventricles (Fig. 2), external hydrocephalus, microcephaly, exencephaly (Fig. 1), asymmetrical hypoplasia of the cerebral hemispheres, microphtalmia, and ectopic eyes.

The skeletal abnormalities were predominantly of minor degree and included increased or decreased number of ribs, and incomplete ossification of

Table 2. Teratogenic effect of lynestrenol on rabbit fetuses[a]

	Control	Dose (mg/kg/day)		
		0.1	0.5	2.5
Number of fetuses examined	76	79[b]	87[c]	11
Number of fetuses examined for malformations in the head	49	48	49	5[d]
Number of fetuses examined for skeletal abnormalities	27	31	38	3
Percentage of litters with malformed fetuses	29	79*	93*	100*
Average % malformed fetuses[e]	12	63*	66*	87*
Number of malformations per malformed fetuses	1.0	1.3	1.4	1.9
Average % fetuses with malformations in CNS/eyes[e]	12	31*	49*	50*
Average % fetuses with malformations in the skeletal system[e]	2	30*	29*	25*
Average % fetuses with malformations in the cardio-vascular system[e]	0	2*	5*	20*

[a] Dead fetuses were excluded from all malformation computations
[b] In addition, three fetuses were dead in utero, two of these were malformed
[c] In addition, ten fetuses were aborted or dead in utero, nine of these were malformed
[d] Three fetuses were lost
[e] Formula is described in Materials and Methods
* Statistically significant higher than the control group (Fisher-Irwin, $p = 0.05$)

the metatarsal, metacarpal, and phalangeal bones (Fig. 3). However, phocomelia and severe distortion of the extremities occurred in four fetuses in the high dose group, all of them had also cranioschisis and exencephaly.

The cardiovascular abnormalities were aortic arch hypoplasia, subaortic stenosis, atrioventricular stenosis, and interventricular septal defects of varying degree. Body weight of the live fetuses was not affected nor was masculinization of the female fetuses observed in any group.

Experiment 2. In this experiment the fetuses were delivered by natural birth at term. Clinical symptoms indicating CNS abnormalities occurred at average percentages of 2 in the lowest and 32 in the high dose group (Table 3). The symptoms comprised ataxia, disorientation, posterior paralysis and rotation of one or both hind limbs. Unilateral anophtalmia was present in one and bilateral

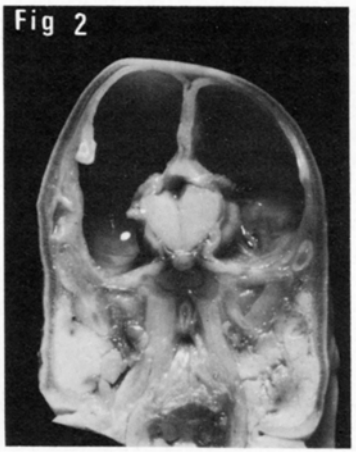


Fig. 1. Multiple malformations including exencephaly, distortion of columna, and malpositioned hindlimb in a rabbit fetus from the high-dose group in Experiment 1

Fig. 2. Internal hydrocephalus affecting lateral ventricles to extreme degree in a rabbit fetus from the high-dose group in Experiment 1

Fig. 3. Alizarin-stained fetus from the medium-dose group in Experiment 1. Delayed ossification of phalangeal bones in forelimbs and of metatarsal and phalangeal bones in hindlimbs

microphtalmia in two weanlings from the high dose group. Multifocal depigmentation of the iris and lenticular opacity was observed in four weanlings which also had symptoms of CNS disturbance from the high dose group, and unilateral glaucoma in one weanling from the same group.

Two weanlings in the low dose group and five in the high dose group were euthanized 3 weeks old for humane reasons. Because of locomotor disturbance

Table 3. Postnatal[a] observations of weanlings of lynestrenol[b] treated rabbits

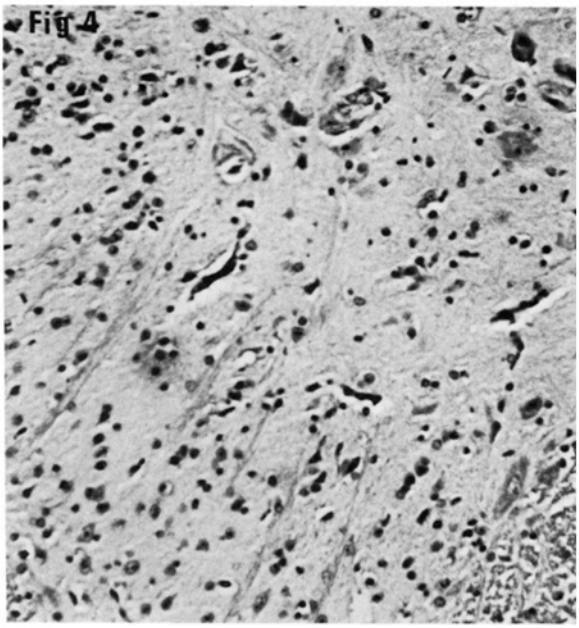
	Control[c]	Dose (mg/kg/day)	
		0.1	0.5
Number of litters	6	6	5
Total number of weanlings	36	36	15
Average number of weanlings per litter	6	6	3
Number of weanlings dead before 4 weeks of age	1	2	5
Average body weight of weanlings (g)[d]	309 ± 17	358 ± 8	394 ± 20
Female/male weanlings	0.80	0.89	1.14
Average % of weanlings with clinical CNS-symptoms[e]	0	2	32*
Total number of weanlings with malformations	3	4	7
Average % of weanlings with malformations[e]	10	12	43*
Average % of malformations in CNS and eyes[e]	0	0	30*
Average % of malformations in the cardiovascular system[e]	8	12	13

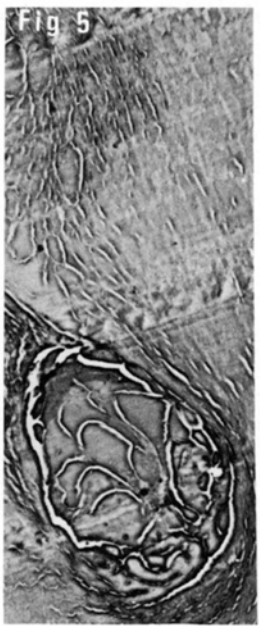
[a] 4 Weeks period
[b] Administered p.o. to the does on days 6–18 of gestation
[c] Received vehicle
[d] At 4 weeks of age
[e] Formula in the text
* Statistically significant ($p = 0.05$) higher than in the control and the low dose group (Fisher-Irwin)

and impaired vision the weanlings were incapable to feed normally. Autopsy findings showed emaciation, underdeveloped and rotated posterior legs, and scoliosis.

Histological examination of the CNS system and the eyes from the weanlings which had been euthanized and from another two which had shown clinical symptoms revealed pathological lesions in the ventral horns of the spinal cord. The lesions consisted of scanty number of neurons, which were partly pyknotic of appearance, some microglial reaction, and proliferated capillaries (Fig. 4).

Histological examination of the eyes with gross lenticular opacity revealed subcapsular and nuclear cataract (Figs. 5 and 6). In addition to epithelial proliferation and "bladder cells" (Fig. 6), degenerated material was observed in the nuclear area (Fig. 5). The lenticular changes were found in combination with retinal foldings and in one case, combined with microphakia, in another case with anterior ectopia of the lens and glaucoma.





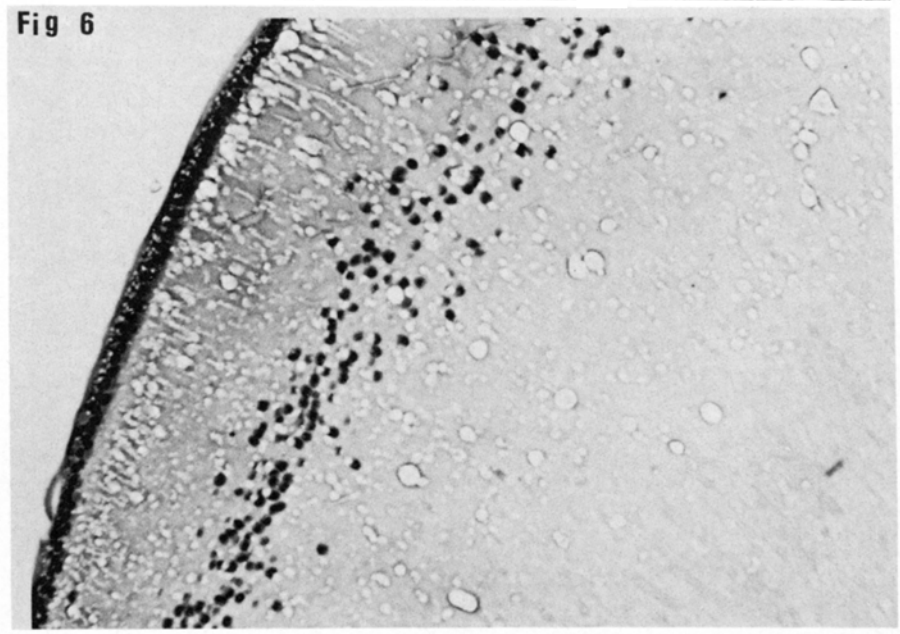


Fig. 4. Ventral horn of the spinal cord of 4 week old rabbit from the high-dose group in Experiment 2. Note almost absence of neurons and proliferation of glia cells. H&E × 170

Fig. 5. Central part of lens of a 4 week old rabbit from the high-dose group in Experiment 2. Note coagulation necrosis of lens material. H&E × 170

Fig. 6. Anterior part of lens of a 4 week old rabbit from the high-dose group in Experiment 2. Note epithelial cell proliferation and vacuolization. H&E × 425

Discussion

The results from the present experiment indicate an embryotoxic and a teratogenic potential of lynestrenol in the Belted Dutch rabbit, as reflected by an increased incidence of post-implantation loss as well as congenital abnormalities following oral treatment of the does. There was a tendency towards increased incidence according to the dosage, though this could not be statistically confirmed with the dose levels chosen. A no-effect level could not be demonstrated with the dose levels which were administered. Takano et al. (1966) examined the teratogenic potential of some synthetic progestogens in rabbit. Their results showed that norethisterone, which like lynestrenol is an ethynyl derivative and has a progestional effect comparable to that of lynestrenol (Overbeck et al. 1962), did not produce teratogenic effects in Japanese albino rabbits. The lethal effect on the rabbit fetuses, however, was significant at a dose of 3 mg/kg. In the same experiment, chlormadinone, which is an acetoxyprogesterone, produced teratogenic effects in rabbits at a dose of 10 mg/kg and in mice at 1 mg/kg. Most of the malformations in the mice were cleft palate, while in the rabbits the malformations were of various types.

The cases of malformations in humans suspected to be associated with steroid sex hormone exposure have also been of varied types (Nora and Nora 1973; Levy et al. 1973; Harlap et al. 1975; Nora et al. 1978). However, the rates of microcephalus were considered high in the report of Goujard and Rumeau-Rouquette (1977), an association between cardiovascular defects and hormone exposure has been suggested, among others, by Heinonen et al. (1977), and, in the study of Janerich et al. (1974), a relationship between hormone exposure and limb reduction deformities was suggested. However, it should be noted that contradictory results are presented in various epidemiological studies, and interpretation of the results is difficult because of methodological differences.

In the present study, the major defects were found in the CNS and in the cardiovascular system. Some of the cases with severe CNS and eye malformations were combined with limb reduction or leg rotation. Multiple defects of this type have not previously been seen in this strain of rabbits in our laboratory.

An effect of lynestrenol administration upon CNS, eye, and leg was also recorded in Experiment 2. Pathological examination supported the clinical symptoms which were observed in the post-natal period. Another experiment has been designed in order to examine the clinical symptoms and the pathological changes more closely.

In attempting to extrapolate these animal teratological data to man, it must be recalled that a considerable dosage difference exists. Even though the highest dose was only about $^{1}/_{2,000}$ of the oral LD_{50} in mice and $^{1}/_{10}$ of the dose producing only slight weight reduction in chronic toxicity testing in rats (Overbeck et al. 1962), the doses were much higher than human therapeutic doses. Possible differences in metabolism should also be considered. For lynestrenol, the general metabolism appears to be similar in rabbits and man, as far as both species appear to metabolize lynestrenol to norethisterone, and to excrete most

of the drug as metabolites in the urine (Kamyab et al. 1968; Mazaheri et al. 1970; Fotherby 1973; Ranney 1977). Furthermore, differences in placental transport and in the fetal development pattern create problems in drawing conclusions from experimental teratology when assessing potential risks to human fetuses.

In conclusion, the results of these experiments indicate a teratogenic effect of lynestrenol in the rabbit. However, it should not be concluded that lynestrenol will show similar adverse effects on human fetuses when administered to women at therapeutic doses.

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