SOME ANTIFERTILITY EFFECTS OF NILEVAR (17 α -ETHYL-19-NORTESTOSTERONE), A PROGESTATIONAL STEROID, IN THE FEMALE GUINEA-PIG*

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Summary. In the adult guinea-pig the progestational steroid Nilevar (17 α -ethyl-19-nortestosterone) alters the pattern of the oestrous cycle, inhibiting opening of the vagina. Ovulation is prevented—as judged by the absence of corpora lutea in the ovaries—but follicles reach a stage of maturity. Gonadotrophins are present in the pituitary gland of the Nilevar-treated guinea-pigs in amounts comparable with those of their oil-treated controls, as shown by tests for total gonadotrophins and for follicular stimulating hormone alone.

It is suggested that in the adult guinea-pig Nilevar does not exert its effect directly on the growing follicle but prevents the action of gonadotrophins on the mature follicle. The results indicate an inhibition of the sudden release of gonadotrophins which probably precedes ovulation. This effect may be at the hypothalamic level of the brain where an inhibition of the gonadotrophin-releasing factors could occur, but the results do not preclude an inhibition at the ovarian level.

INTRODUCTION

Progesterone and certain hormonally related steroids are known to inhibit fertility in the female mammal, but, as yet, little is known as to the site and mode of action of these antifertility substances. In the guinea-pig, Loeb (1911) described inhibition of ovulation by the presence in the ovary of functional corpora lutea and Papanicolaou (1926) obtained inhibition of ovulation by the injection of extracts of corpora lutea. In 1956 Pincus, Chang, Zarrow, Hafez & Merrill showed that certain 19-norsteroids, including Nilevar, could inhibit ovulation in rats and rabbits. Rothchild (1962) suggested that progesterone inhibits the release but not the formation of pituitary folliculotrophin (follicular stimulating

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hormone and luteinizing hormone), as shown by the assays of extracts of pituitary tissue taken from rats treated with the hormone or from those made pseudopregnant—presumably secreting progesterone. Lakshman & Nelson (1963) observed no outstanding microscopic differences between the pituitary glands of norethynodrel-treated and untreated rats; the steroid did not seem to exert its effect directly on the anterior pituitary gland but possibly on the 'neurohumoral system'. Saunders (1964), however, found that norethynodrel and testosterone propionate 'inhibited' pituitary gonadotrophins in ovariectomized rats, whilst Nilevar 'was less effective in suppressing gonadotrophin accumulation'.

In the present investigation some of the effects of Nilevar on the oestrous cycle, the ovary and the anterior pituitary gland in the adult guinea-pig have been observed. Since antifertility substances are in use clinically, most of the experiments were performed in the normal animal so as to avoid, as far as possible, the effects of hormonal imbalance. The guinea-pig was chosen for these investigations as, in contrast to other small laboratory mammals which ovulate spontaneously, it resembles man in having a fully functional corpus luteum after ovulation.

MATERIAL AND METHODS

Albino guinea-pigs of the Hampstead strain, weighing on average 650 g at the beginning of the experiment, were used. The animals were fed on diet S.G.I. (Scientific Animal Service, Elstree, Hertfordshire) supplemented with hay and fresh cabbage to provide them with a source of ascorbic acid. The vaginal closure membrane, characteristic in the guinea-pig, was examined daily: this membrane allows opening of the vagina for a few days only in each oestrous cycle, and remains closed throughout the anoestrous period. The guinea-pig has an irregular cycle until it is 3 months old, which approximates, in the strain used, to a body weight of about 500 g. Up to this time the oestrous cycle is prolonged, due to the period of oestrus being longer than in the adult pig. At least two complete cycles were observed in each animal before any treatment was started and only those animals which maintained a regular pattern, when adult, were used in the experiments. The first day the vagina was fully open was counted as Day 0 and injections were begun a day or two after the vagina had closed. Control guinea-pigs were killed on the 5th day and 6th day (0+5 to 6) and Nilevar-treated animals on the day estimated to be the 5th or 6th day for each individual pig—since, in these animals, the vagina did not open at the normal time.

Dissections

All guinea-pigs were killed with chloroform at the end of the experiment and the organs dissected as quickly as possible. The uteri were gently pressed between blotting paper to remove any fluid from the lumen before being put into specimen bottles for weighing, by difference, on a Stanton balance. The ovaries were weighed in pairs on a 250 mg torsion balance.

Histology

The paired ovaries were fixed in 10% formol-saline and processed for histological examination. Serial sections were cut at $7\ \mu$ and every tenth section was stained with haematoxylin and eosin.

Ovariectomy

Bilateral ovariectomies were performed by the lumbar approach under pentobarbitone anaesthesia (0.1 ml/150 g body weight). In one or two cases this was supplemented with ether anaesthesia, the ether being applied by means of a wire mesh mask lined with cotton wool. Incisions were made through the skin and muscle, and the ovary and oviduct raised into view so that, after ligaturing the ovarian artery and vein, the ovary could be freed from surrounding tissue and removed. The pigs were returned to their cages whilst still under the influence of anaesthesia. They had fully recovered from the operation by the next day.

Assays of pituitary gonadotrophins

Two tests were performed on immature albino mice (National Institute for Medical Research) in order to find out if the pituitary of guinea-pigs—killed after a course of steroid injections—had a store of gonadotrophins comparable in amount with those of the oil-injected control animals. Glands dissected out at the end of the experiment were placed in bijoux bottles and kept refrigerated at -20°C until a sufficient number had been collected for assay.

Total gonadotrophins. For determining the gonadotrophin content of the pituitary a modification of the methods of Loraine & Brown (1959), Claringbold & Lamond (1957) and Bahn, Lorenz, Bennet & Albert (1953) was adopted. Three groups of mice were used; one group was injected with homogenates of pituitaries from the Nilevar-treated guinea-pigs, one with pituitary homogenates from oil-control guinea-pigs and one group was injected with saline only. The pituitaries were homogenized with a small volume of tap water and sufficient physiological saline was added to provide a suspension of 1 pituitary/0.1 ml for each mouse. The injections were made subcutaneously through the loose dorsal skin and each mouse received two injections of the freshly prepared homogenate in saline at 24-hourly intervals. Fifty hours after the first injection the mice were killed and the uteri dissected out, gently pressed between blotting paper to remove fluid from the lumen, and weighed.

Follicular stimulating hormone (FSH) augmentation test. The methods of Brown (1955) and Steelman & Pohley (1953) were modified for this test where human chorionic gonadotrophin (HCG) is used with the test material in one group of animals and alone in a control group. Any increase in ovarian weight in the animals treated with the test material over that of the controls is due to the effect of the test material. Three groups of mice were used. One was injected with HCG and an anterior pituitary homogenate from oil-injected guinea-pigs, one with HCG and an anterior pituitary homogenate from Nilevar-injected guinea-pigs and one, a control group, was given HCG only. The dose of HCG used throughout was 20 i.u. and the injection volume for all groups was 0.2 ml/mouse—injected subcutaneously as before. Two thirds of a pituitary were injected

into each mouse receiving an homogenate. Three injections were given at 24-hr intervals. The mice were killed 72 hours after the first injection and the ovaries and uteri weighed.

Steroids

Nilevar (G. D. Searle & Co., Ltd, High Wycombe, Buckinghamshire) in 0.2 ml oil (40% ethyl oleate and 60% arachis oil) was injected subcutaneously into the guinea-pigs, using the left and right flank on alternate days. Oil injections into the scruff of the neck of adult guinea-pigs are not satisfactory as there is a pad of fat which interferes with absorption. As each experiment was to last for some weeks a sufficient quantity of the steroid was dispensed into sterile vaccine bottles and rubber caps (autoclave sterilized) were fitted on to the top of the bottle. In most of the experiments the dosage was 2 or 3 mg/day. At these concentrations Nilevar can be dissolved in oil over a water bath at 70° C. In the two assays for pituitary gonadotrophins a high dosage of 4 mg or over was used. For these, a suspension was made by first grinding the chemical to a very fine powder with a pestle and mortar and then rotating the chemical with the oil medium in a ball mill at a moderate speed for 4 hr. The dosage related to a particular experiment is recorded in the results.

Oestradiol benzoate (British Drug Houses Oestroform) in ampoules containing 5 mg/ml was diluted with a mixture of ethyl oleate (40%) and arachis oil (60%) to give a concentration of 100 µg/0.2 ml.

RESULTS

Oestrous cycle

In order to establish a pattern of the normal oestrous cycle in the guinea-pig, examinations of the vaginal membrane in a group of untreated animals were made at the same time each day for 2 months. The oestrous cycle was divided into two parts—the long anoestrous period when the vagina remained closed and the shorter period at or around oestrus when the vagina was fully open or only partially sealed by the closure membrane. Generally a slight reddening of the mammary glands, reddening and enlargement of the nipples and swelling of the external genitalia could be seen just before rupture of the membrane. In the group studied, the average duration of the oestrous cycle was 16 days, the vagina being fully open for 2 to 3 days and partially open for a day or two. After this period of observation the guinea-pigs were killed at various stages of the cycle and the uteri and ovaries dissected and weighed. The abrupt rise in uterine weight which occurred just before oestrus is shown in Text-fig. 1 where the variations in uterine weight, in relation to the different stages of the cycle, are recorded graphically. The lowest weights occurred when the uterus was quiescent—in the mid-anoestrous period and for a few days following.

Effects of Nilevar on the pattern of the oestrous cycle

Daily injections of Nilevar at the dosage of 2 mg altered the pattern of the oestrous cycle as shown by the observations on the vaginal closure membrane depicted in Text-fig. 2. Vaginal opening was inhibited throughout the injection

period of 2 months in three out of six guinea-pigs; in the other three animals the membrane was occasionally partially ruptured but the pattern of a normal cycle was not obtained.

The group of guinea-pigs injected with the oil medium only for 5 weeks had oestrous cycles similar in pattern to those of the untreated animals. The uterine weights of a group of eight guinea-pigs treated with 3 mg/day of Nilevar (mean value 1.0 ± 0.02 [S.D.] g) were within the range of weights shown by those of the two groups of six uninjected pigs (mean value 1.0 ± 0.02 [S.D.] g) and eight

TEXT-FIG. 1. Relationship of uterine weight to different parts of the guinea-pig oestrous cycle. Each column represents the uterine weight of one guinea-pig. 0 is the first day the vagina was fully open, 2 is 2 days after the vagina was fully open, etc.; 16 would be the first day of the next cycle when the vagina would be expected to be fully open.

pigs injected with the oil medium (mean value 0.92 ± 0.14 [S.D.] g), killed in the anoestrous period.

The ovarian weights (average of paired ovaries) of the steroid-treated guinea-pigs (mean value 39.4 ± 7.1 [S.D.] mg) showed variations comparable with those of the guinea-pigs injected with the oil medium only (mean value 46.7 ± 6.8 [S.D.] mg) and with those of the untreated pigs (mean value 37.9 ± 7.9 [S.D.] mg).

Ovariectomy. In order to find out a little more about the opening and closing mechanism of the vaginal membrane of the guinea-pig and, in particular, to see if the vagina closed spontaneously and not under the influence of any hormones, two groups of five animals were ovariectomized, one during the anoestrous part of the cycle—the other on the first day that the vagina was fully open. Daily examinations of the vaginal membrane were made and it was found that after ovariectomy the membrane no longer ruptured periodically—the vagina remaining closed indefinitely. When the ovaries were removed on the first day that the membrane was completely ruptured, the vagina remained open for

a further day or two before closing—giving a pattern similar to that in the unoperated animal. At autopsy (8 weeks after ovariectomy) the uteri of the guinea-pigs were pale and small. The uterine weights (mean value 0.47 ± 0.11 [S.D.] g) were considerably less than those of the normal anoestrous pigs.

Oestradiol benzoate. In a preliminary experiment, five unoperated guinea-pigs were given daily injections of oestradiol benzoate ($100 \mu\text{g}/\text{day}$) so that the effect of an oestrogen derivative on the vaginal membrane could be studied. The vagina opened a week or so after the commencement of treatment (Text-fig. 3)

TEXT-FIG. 2. Vaginal examinations of guinea-pigs injected daily with Nilevar (2 mg) Each division represents 1 day. The injections were started the day after the vagina closed and continued until the day before autopsy. White, vagina closed; black, vagina open; cross-hatched, vagina half open.

and remained open in four out of five of the group throughout the course of treatment, closing again a few days after the cessation of injections.

Five ovariectomized guinea-pigs were observed for 30 days after the operation and then injected with one single dose of $100 \mu\text{g}$ of oestradiol benzoate and a week later two further injections of $100 \mu\text{g}$ of the steroid were given with an interval of 24 hr. A single dose of the oestradiol benzoate was not sufficient to cause the membrane to rupture completely (in one animal the vagina remained closed), but the same dosage given on two consecutive days was effective (Text-fig. 4). In all animals the vagina closed again after a few days.

Effects of Nilevar on the ovary

Histological sections of ovaries of six guinea-pigs treated with 2 mg/day Nilevar for 6 weeks showed no recent corpora lutea nor any corpora albicantia. Comparison of the follicles in these ovaries with those of the ovaries of the series of uninjected animals suggests that the follicles had grown to a certain stage of maturity but that the final stage of ripening and rupture was not attained (see Plates 1 and 2).

PLATE 1

Photomicrographs of sections of ovaries from Nilevar-treated and untreated guinea-pigs. $\times 30$.
(a) Ovary of uninjected animal showing two recent corpora lutea and one of previous cycle. Maturing and atretic follicles. (b) Ovary from animal injected with 2 mg Nilevar daily for 2 months showing maturing and atretic follicles. There are no corpora lutea.

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Photomicrographs of sections of ovaries from Nilevar-treated and untreated guinea-pigs. $\times 80$.

- (a) Ripe follicle from ovary of untreated animal showing increase in size of the granulosa cells which are no longer packed in rows. There is a marked increase in the vascular supply to the internal theca.
 (b) Mature follicle from ovary of animal injected with 2 mg Nilevar daily for 2 months. Granulosa cells are still packed on rows. There is little increase in the vascular supply to the internal theca.

Histological sections of ovaries from a control group of guinea-pigs injected with the oil medium showed follicles and corpora lutea comparable with those of uninjected animals killed at a similar time of the oestrous cycle.

TEXT-FIG. 3. Vaginal examinations of guinea-pigs injected daily with oestradiol benzoate (100 µg). Each division represents 1 day. Injections begun after oestrus. White, vagina closed; black, vagina open; cross-hatched, vagina half open.

TEXT-FIG. 4. Daily vaginal examinations of ovariectomized guinea-pigs subsequently treated with oestradiol benzoate. The arrows on the left-hand side indicate the day of operation, those on the right-hand side the days of injection of oestradiol benzoate. Each division represents 1 day. White, vagina closed; black, vagina open; cross-hatched, vagina half open.

Effects of Nilevar on pituitary gonadotrophins

Total gonadotrophins. Three groups of seven immature mice were used in this assay. The group of mice injected with homogenates of pooled pituitaries from guinea-pigs treated with a suspension of Nilevar (5 or 10 mg/day) for 3½ weeks had uterine weights (mean value 17.8 ± 2.8 [S.D.] mg, $P < 0.001$) comparable with those of mice injected with homogenates from a group of oil-treated

guinea-pigs (mean value 16.6 ± 3.6 [S.D.] mg, $P < 0.02$). The weights of the uteri of both groups of mice are significantly higher than those of the saline control group (mean value 4.7 ± 0.88 [S.D.] mg).

FSH augmentation test. In the FSH augmentation test (Table 1) where the ovarian and uterine weights of groups of immature mice injected with anterior pituitary homogenates and HCG are compared with those of the control group

TABLE 1
FOLLICULAR STIMULATING HORMONE TEST IN THE IMMATURE MOUSE

Ovarian weights (paired ovaries) (mg)			Uterine weights (mg)		
Controls : saline and HCG only	HCG and pituitary homogenate from oil-treated guinea-pigs	*HCG and pituitary homogenate from Nilevar-treated guinea-pigs	Controls : saline and HCG only	HCG and pituitary homogenate from oil-treated guinea-pigs	*HCG and pituitary homogenate from Nilevar-treated guinea-pigs
7.1	10.6	14.6	23.8	30.6	35.0
9.4	14.4	16.7	26.6	34.6	40.5
8.0	12.2	10.7	29.6	36.8	34.8
9.3	8.6	13.8	23.9	52.4	37.8
9.4	7.6	12.2	27.5	46.4	36.0
8.0	15.6	9.7	27.2	33.7	38.7
8.3	11.6	12.7	26.3	30.2	37.5
8.2	—	10.4	21.5	—	35.4
<i>t</i> <i>P</i>	<i>t</i> = 2.87 0.01 < <i>P</i> < 0.02	<i>t</i> = 4.66 <i>P</i> < 0.001	<i>t</i> <i>P</i>	<i>t</i> = 3.86 <i>P</i> = 0.002	<i>t</i> = 9.66 <i>P</i> is minute
Mean 8.5 S.D. ± 0.83	11.5 ± 2.9	12.6 ± 2.4	Mean 25.8 S.D. ± 0.9	37.8 ± 2.8	37.0 ± 0.7

Human chorionic gonadotrophin (HCG), large dose eliminates effect of LH in homogenates.

* Pooled pituitaries from guinea-pigs which had received 4, 2 or 1 mg/day Nilevar for 2 months.

which received injections of HCG alone, a significant increase in these weights is seen. The uterine and ovarian weights of those mice treated with pooled pituitary homogenates from guinea-pigs treated with 4, 2 or 1 mg/day for 2 months are comparable with those weights in the mice receiving homogenates from the oil-control guinea-pigs.

DISCUSSION

The results presented show that at the dose levels used, Nilevar altered the pattern of the oestrous cycle in the adult guinea-pig, the vagina failing to open during the injection period. Ovulation was inhibited as shown by the absence of corpora lutea in the ovaries of treated animals—but the results of the pituitary assays suggested that gonadotrophins were being stored in these animals.

The possibility of a direct action of this progestational steroid on the vaginal membrane has been investigated. However, the series of guinea-pigs ovariectomized at any point during anoestrus, and those operated on the first day the

vagina was open, showed that, in the untreated animals, the membrane closes spontaneously and not under the influence of ovarian hormones. Oestradiol benzoate injected into ovariectomized guinea-pigs 30 days after the operation caused the vaginal membrane to rupture and it was found that 100 μg injected on two consecutive days were required to do this. This suggests that Nilevar in some way had prevented the formation or liberation of sufficient oestrogen in the ovary to effect rupture of the vaginal membrane. It is probable that a basic amount of oestrogen is secreted in the ovary throughout the oestrous cycle—an amount sufficient to maintain the accessory sex organs and perhaps also to aid the maturation of the follicles. The uterine weights of the group of guinea-pigs killed 8 weeks after ovariectomy were considerably lower than those of the groups of uninjected animals killed in the mid-anoestrous period (the period of lowest uterine weight in the uninjected animal) indicating that, in the absence of ovarian hormones, and in particular oestrogens, atrophy of the uterus occurs. In the Nilevar-treated guinea-pigs the uterine weights obtained at autopsy were within the range of weights obtaining in the uninjected guinea-pigs—suggesting that Nilevar had not inhibited this basic secretion of oestrogens.

Ovarian histology of the Nilevar-treated guinea-pigs showed that the follicles were capable of maturing, and various stages of growth up to just before ovulation were observed, indicating that Nilevar does not exert its effect directly upon the growing follicle, but rather that it prevents the action of FSH. If the follicle were not at a suitable stage of development it is probable that luteinizing hormone (LH) could not exert its effect.

The results of the assays for pituitary gonadotrophins showed that the pituitaries of the Nilevar-treated guinea-pigs had a gonadotrophin content comparable with that of their oil-injected controls. This suggests that Nilevar had not inhibited the pituitary directly—since gonadotrophins had obviously been formed and stored—but possibly that it had inhibited the release of these hormones by acting at some higher level in the brain—probably in the hypothalamus. The test for total gonadotrophins is a measure of the combined stimulating action of FSH and LH on the ovary, increasing its secretion of hormones (in particular oestrogens) which, in turn, act upon the uterus. The ovarian augmentation test is specific for FSH (Schmidt-Elmendorff, 1964)—as is also the augmentation of uterine weight. This has been shown recently by Igarashi & McCann (1964) who devised a similar test.

In the normal guinea-pig the abrupt increase in uterine weight, which occurred just before oestrus, was probably due to the rapid increase in circulating oestrogens, causing a rise in intracellular fluid. This would cause a rapid rise in intracellular pressure which would also occur in the vagina—so causing rupture of the vaginal membrane. The fact that the vaginal membrane failed to rupture in these Nilevar-treated guinea-pigs was perhaps also due to inhibition of the marked increase in the release of FSH which may occur just before ovulation. This inhibition would, in turn, prevent the rise in oestrogen output from the ovary.

Little information is, as yet, available as to the mechanism of action of antifertility compounds. Greenwald (1964) has found that one large single dose of norethynodrel inhibited ovulation in three out of five of a group of hamsters.

The ovaries of the animals which did not ovulate contained large normal pre-ovulation follicles. Overbeek & de Visser (1964) have shown that Lynestrenol (17-ethinyl-estr-4-ene-17 β -ol) and 6-methyl Lynestrenol, i.e. two compounds unrelated to progesterone, inhibited ovulation in rats: Lynestrenol was effective only if administered early in the cycle but the methyl compound could prevent ovulation if given as late as 1 day before the expected release of LH. They concluded that Lynestrenol probably inhibited ovulation by preventing the release of FSH from the pituitary gland, and the methyl compound inhibited the release of LH. Holmes & Mandl (1962) showed that norethynodrel altered the oestrous-cycle pattern in adult female rats and inhibited ovulation in some, but not all, of the animals treated. They suggested that the steroid had affected 'elaboration and/or release' of FSH as well as LH. Saunders & Drill (1958), however, investigating the effect of the same drug, thought that gonadotrophin secretion had been depressed. Epstein, Kupperman & Cutler (1958) suggested that some of the nortestosterone derivatives (including Nilevar) are pituitary inhibitors, and Kincl, Ringold & Dorfman (1961), using progesterone and testosterone, postulated an 'antipituitary' activity of these substances.

Altogether the evidence on the action of these antifertility substances points to an inhibition of the release of the pituitary gonadotrophins rather than a direct action upon the ovary. This could be in accord with the conclusions derived from the present experiments in the adult guinea-pig that Nilevar does not exert its effect directly on the growing follicle, but prevents the action of gonadotrophins on the mature follicle—possibly by inhibition at the hypothalamic level of the gonadotrophin releasing substances. The results presented, however, do not preclude an inhibition of gonadotrophins at the ovarian level.

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