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Elongation of oestrous cycle in the guinea-pig following subcutaneous or intra-uterine administration of indomethacin

E. W. HORTON AND N. L. POYSER

Deparment of Pharmacology, University of Edinburgh, 1 George Square, Edinburgh EH8 9JZ

Summary

1. Guinea-pigs injected subcutaneously with 10 mg indomethacin twice daily, from day 7 of the oestrous cycle, had their oestrous cycles significantly lengthened ($P<0.001$) by about 3 days.

2. When the indomethacin was incorporated into a slow-release preparation and placed in the uterus (33 mg per horn), the oestrous cycle lengths of these guinea-pigs were greatly prolonged and approached that produced by hysterectomy.

3. The measurement of corpora luteal size and progesterone levels in some of the guinea-pigs autopsied on day 47 of the extended cycle showed functional corpora lutea to be present.

4. It was apparent that indomethacin had prolonged the life span of the corpus luteum. This would account for the increase in the length of the oestrous cycle.

5. Recent investigations suggest that normal corpora luteal regression, with the termination of the oestrous cycle, is induced by prostaglandin $F_{2\alpha}$ released from the uterus on day 15. Since indomethacin prevents prostaglandin biosynthesis, it is probable in these experiments that this drug had prevented luteal regression by inhibiting the biosynthesis by, and release from, the uterus of prostaglandin $F_{2\alpha}$ in luteolytic concentrations.

6. These results provide further evidence that the uterine luteolytic hormone in guinea-pigs is prostaglandin $F_{2\alpha}$.

Introduction

There is now substantial evidence to support the conclusion that the uterine luteolytic hormone in the guinea-pig is prostaglandin $F_{2\alpha}$ (Blatchley & Donovan, 1969 ; Poyser, Horton, Thompson & Los, 1970, 1971 ; Blatchley, Donovan, Poyser, Horton, Thompson & Los, 1971 ; Blatchley, Donovan, Horton & Poyser, 1972 ; Poyser, 1972). If this is true, inhibition of prostaglandin $F_{2\alpha}$ biosynthesis in the uterus should prolong the life of the corpus luteum and so increase the length of the oestrous cycle. Indomethacin inhibits prostaglandin biosynthesis by the spleen *in vitro* (Vane, 1971) and also in homogenates of guinea-pig uterus (Poyser, 1972). In this paper we report the results of experiments in guinea-pigs in which indomethacin, either injected subcutaneously or, when given by intra-uterine implanta-

tion, increased the length of the oestrous cycle. Some of these results have been briefly reported (Poyser, 1973).

Methods

Vaginal smears

Virgin guinea-pigs weighing 500–800 g were smeared daily by a lavage technique. The smears were examined for the presence of leucocytes, epithelial cells and cornified cells by light microscopy (60 times magnification) without staining. The first day of the cycle was taken as the day preceding the post-ovulatory influx of leucocytes when cornification of the vagina was maximal.

Indomethacin injections

Indomethacin dissolved in 0·5 ml polyethylene glycol was injected subcutaneously at 12 hourly intervals from the 7th day of the cycle until the 1st day of the next cycle, at two dose levels (4 and 20 mg/day). Control guinea-pigs were similarly injected with 0·5 ml polyethylene glycol.

Intra-uterine implants

Implants (15 mm long, 2 mm maximum diameter, weighing 25 mg) were prepared by the incorporation of indomethacin powder into paraffin wax (m.p. 43–46° C) in the ratio of 1:2 parts by weight. For control purposes similar implants containing paraffin wax alone were prepared.

On the 10th day of the cycle, guinea-pigs were anaesthetized with pentobarbitone sodium, 30 or 35 mg/kg, injected intraperitoneally. Anaesthesia was supplemented by intradermal and subcutaneous infiltration of 1% procaine hydrochloride down the mid-line of the abdominal wall. An incision about 5 cm long was made in the mid-line of the middle and lower abdomen. The abdominal contents were retracted to one side to reveal the cervix and vagina. A loose ligature was placed around the upper vagina. Whilst pulling gently on the ligature, an incision was made into the lower cervical region of the uterus. A blunt metal probe (1·5 mm diameter) was passed through the cervix into the lumen of each horn in turn to check its patency. A piece of polyethylene tubing (external diameter 2·6 mm) was inserted into one uterine horn. Four implants were placed in each horn, being pushed with the metal probe, through the lumen of the polyethylene tubing, which was withdrawn as every implant was inserted into the horn. When the fourth implant was in position, the tubing was removed and a mersilk ligature was tied around the vaginal end of the uterine horn so as to retain the implants but not to obliterate the lumen entirely. The incision in the cervix was sutured with fine catgut and the loose ligature around the vagina was removed. The muscle layers were closed with continuous catgut suture and the skin with interrupted mersilk sutures. The skin sutures were removed 5 to 7 days post-operatively. The guinea-pigs were injected intraperitoneally with tetracycline (5 mg) twice daily for 5 days post-operatively.

Plasma progesterone levels and autopsy examination

At the end of each implantation experiment, the animals were stunned by a blow on the head and blood collected into heparinized tubes by severing the large vessels

of the neck. Assays of plasma progesterone levels were kindly performed blind by Dr. R. B. Heap and Mrs. Susan Broad using the competitive binding methods of Challis, Heap & Illingworth (1971).

The abdomen was opened and the uterine horns and ovaries removed intact. The ovaries were dissected free of connective tissue and stored at 4° C in formol saline. After checking that the uterine ligatures were in place, the horns were incised and the intra-luminal fluid which had accumulated was collected and measured. The implants were then removed, dried and weighed. The horns were opened and the condition of the endometrium noted. A section was taken for histological examination. After weighing, the horns were homogenized in Tyrode solution and incubated.

Histological examination of the ovaries

This was kindly performed by Dr. K. P. Bland, who measured the size and number of corpora lutea in each ovary, using the method of Bland & Donovan (1966). On the basis of these measurements and of the general appearance of the ovaries he was able to assess the functional state at the time of death without knowing the origin of the samples he was examining.

Prostaglandin formation by uterine tissues

The uterine homogenates in Tyrode solution were incubated for 90 min at 37° C. The prostaglandins formed were solvent extracted and then separated by methods previously described (Poyser, 1972). The prostaglandin E and F content was measured by bioassay on the rat fundal strip in terms of prostaglandin E_2 and $F_{2\alpha}$ and the identity of the prostaglandins confirmed by gas chromatography–mass spectrometry. Similarly, the fluid collected from the uterus was analysed for the presence of prostaglandins.

Estimation of indomethacin in paraffin wax implants

Implants removed at autopsy, after weighing, were dissolved in chloroform and their indomethacin content was determined by measuring absorbance at 322 nm.

Results

Effects of subcutaneous injection of indomethacin on the length of the oestrous cycle

The mean length of the oestrous cycle in guinea-pigs injected subcutaneously with indomethacin (20 mg/day) from the 7th day of the cycle until the onset of the next cycle was $19{\cdot}3 \pm 0{\cdot}5$ days ($n=12$). This result was significantly greater ($P<0{\cdot}001$) than the length of cycles of the same guinea-pigs before and after indomethacin treatment. It was also significantly greater than the cycles of control animals injected with vehicle alone, or than those of animals injected with a lower dose of indomethacin (4 mg/day). These results, summarized in Table 1, indicate that indomethacin at the higher dose, but not the lower dose, has a significant effect in increasing the length of the oestrous cycle. However, they give no indication as to the site at which indomethacin is acting. Moreover, this dose of indomethacin was toxic. Two animals at the 20 mg/day level died after 11 days of

TABLE 1. *Effect of indomethacin on oestrous cycle length in guinea-pigs following subcutaneous injections*

Dose of indomethacin (mg/day, sc)	Oestrous cycle length (days) Before treatment	During treatment	After treatment
20	16·7±0·3 (n=12)	19·3±0·5* (n=12)	16·0±0·2 (n=12)
4	17·8±0·5 (n=4)	17·3±0·3 (n=4)	17·0±0·4 (n=4)
Control	16·9±0·4 (n=8)	16·1±0·3 (n=8)	15·8±0·3 (n=8)

* Significantly lengthened ($P<0·001$); n=Number of oestrous cycles per group.

injections. Since we were concerned with inhibiting uterine production of prostaglandin $F_{2\alpha}$, we devised a method for introducing a high local concentration of indomethacin into the uterus.

Effects of intra-uterine administration of indomethacin on the length of the oestrous cycle

In six guinea-pigs, implants of paraffin wax containing indomethacin were placed in each uterine horn, whilst in the six control animals paraffin wax implants alone were inserted. The results are shown in Table 2. The length of the three oestrous

TABLE 2. *Effect of intra-uterine implants of indomethacin on the length of oestrous cycles in guinea-pigs*

Implant	Length of oestrous cycle (days) Before implantation	Cycle in which implants were inserted	After implantation
Indomethacin and paraffin wax	16·4±0·2 (n=18)	75, 70, 51 >47, >47, >47	see text
Paraffin wax (control)	16·6±0·2 (n=18)	19·5±1·0 (n=6)	14·8±0·2 (n=18)

cycles before the operation in each of the 12 animals was estimated. The mean lengths of 16·4±0·2 (n=8) and 16·6±0·2 (n=18) days for the two groups were not significantly different. In the six guinea-pigs receiving paraffin wax implants the length of the cycle in which the operation was performed increased to 19·5± 1·0 days and the mean of the three subsequent cycles was 14·8±0·2 days. In contrast, in each of the guinea-pigs receiving intra-uterine indomethacin implants, the cycle in which the operation was performed was in excess of 47 days. Three of these guinea-pigs were killed on the 47th day and the remaining three came into oestrus after cycle lengths of 75, 70 and 51 days. The first of these three had a subsequent cycle of 49 days and was then killed on the 8th day of the next cycle. The second was killed on the 9th day of the first post-operative cycle and the third had two post-operative cycles of 17 days before being killed on the 9th day of the next cycle. It is clear from these results that intra-uterine administration of indomethacin causes a great prolongation of the oestrous cycle in guinea-pigs, an effect which cannot be accounted for by the implantation technique itself.

Assessment of luteal function

The six control animals receiving paraffin wax implants were allowed to come into oestrus four times following the operation. During the next cycle three were

killed on the 8th day and three were killed on the 14th or 15th day of the cycles at times corresponding to low and high uterine prostaglandin production respectively (Poyser, 1972). Of the six animals with indomethacin implants three were killed 37 days after the operation (on the 47th day of the cycle) and the three which came into oestrus were killed on the 8th or 9th day of a subsequent cycle. Luteal function was assessed independently and blindly by two workers using histological examination and progesterone measurement. The results are shown in Table 3.

TABLE 3. *Tests of luteal function in guinea-pigs with intra-uterine implants*

Guinea-pig	Day of cycle killed	Plasma progesterone (ng/ml)	Size of C.L. mm³	Uterine fluid Volume (ml)	Uterine fluid PG content (ng/ml)		Uterine weight (g)	PG production ng/100 mg tissue	
					$F_{2\alpha}$	E_2		$F_{2\alpha}$	E_2
Indomethacin implants									
1	8	2·37	3·77	4·8	<6·3	<4·2	2·9	34·5*	4·3
2	9	3·14	2·88	5·9	<3·4	<5·4	3·6	28·8*	3·2
3	9	3·60	3·11	1·5	<20·0	<13·3	2·1	33·3*	5·2
4	47	8·77	4·28	1·0	<20·0	<10·0	2·2	34·7*	6·9
5	47	3·78	4·25	3·5	80·0*	<5·7	2·6	95·1*	4·9
6	47	8·90	6·00	3·5	<11·4	<5·7	2·3	143·5*	4·8
Paraffin wax implants									
7	8	5·51	3·07	2·8	<7·1	<11·4	2·1	29·2*	4·4
8	8	4·55	2·00	19·0	<2·1	<1·0	5·5	45·4*	6·5
9	8	6·84	2·40	25·0	<1·6	<1·3	6·7	43·3*	5·2
10	15	0·36	0·95	63·0	30·3*	<2·6	17·2	72·7*	13·3*
11	14	0·27	0·79	13·0	5·4	<3·1	7·0	157·8*	35·9
12	14	0·62	0·55	8·5	<7·0	<3·7	3·7	200·0*	7·5

*Confirmed by gas chromatography-mass spectrometry.

The plasma progesterone levels of animals treated with indomethacin and killed on day 8 or 9 of the cycle were between 2·4 and 3·6 ng/ml and the mean size of the corpora lutea ranged from 2·9 to 3·8 mm³. In three animals treated with indomethacin which had still not come into oestrus on the 47th day the plasma progesterone levels were 8·8, 3·8 and 8·9 ng/ml and the mean sizes of their corpora lutea were 4·3, 4·3 and 6·0 mm³. In the last two, the corpora lutea resembled those seen in hysterectomized guinea-pigs.

In the control animals killed on day 8, plasma progesterone levels ranged from 4·6 to 6·8 ng/ml and the corpora lutea sizes from 2·0 to 3·1 mm³. In those killed at the end of the cycle plasma progesterone levels were within the range 0·3 to 0·6 ng/ml. The corpora lutea sizes ranged from 0·6 to 1·0 mm³ and had clearly regressed.

Autopsy examination of uterus

All uteri were hypertrophied and contained intraluminal fluid ranging from 1·5 to 63 ml (Table 3). The fluid was slightly alkaline (pH 8). The endometrium was vascular and hypertrophied. On histological examination the endometrium was slightly thinner than normal, the endothelium was thicker and there were fewer gland cells. These changes are characteristic of the fluid-distended uterus. There was no difference between indomethacin-treated and control animals.

Prostaglandin production by guinea-pig uterine homogenates

Although uterine fluid was found in all animals, biological activity corresponding to prostaglandin $F_{2\alpha}$ was detected only in three fluids. In two of these (Nos 5 and 10, Table 3), the identity of the material was established as prostaglandin $F_{2\alpha}$ by combined gas chromatography-mass spectrometry. No prostaglandin E_2 could be detected in the fluid from any animal (Table 3).

On the other hand, prostaglandins were produced when homogenates of the uteri were incubated. In the control animals the level of production was significantly greater at the end of the cycle than at mid-cycle, confirming the observations of Poyser (1972). In the indomethacin-treated animals, prostaglandin production at mid-cycle was similar to that in mid-cycle animals with paraffin wax implants. On the other hand, uterine production of prostaglandin $F_{2\alpha}$ in two of the three animals killed on the 47th day of the cycle was elevated. The prostaglandin $F_{2\alpha}$ was identified by methods previously reported (Poyser, 1972). Prostaglandin E^2 production was considerably smaller but showed some increase towards the end of the cycle (Table 3). In most samples the levels were too low to allow identification. In one sample, however, there was sufficient to identify it as prostaglandin E_2 by gas chromatography–mass spectrometry after conversion to the methyl ester trimethylsilyl ether of prostaglandin B_2.

Discussion

The plasma half-life of indomethacin following intravenous injection in the guinea-pig is short (20 min) and the plasma levels are low. Moreover the uterus takes up only very small amounts (Hucker, Zacchei, Cox, Brodie & Cantwell, 1966). These facts may account for the high dose levels (20 mg/day) required to cause a significant increase in the length of the oestrous cycle when injected. Certain tissues can concentrate indomethacin above plasma levels, notably the small intestine, and in our experiments two animals actually died with gastro-intestinal perforations following 11 days of treatment.

The increase in cycle length following indomethacin injections is compatible with the hypothesis that indomethacin blocks formation of luteolysin in the uterus and thus prolongs the life of the corpora lutea. Similar results were obtained by Marley (1972) following daily oral administration of indomethacin (40 mg/kg beginning on day 11. It is probable that in both these studies prostaglandin synthesis was blocked at many other sites and that these could have contributed to the overall picture. However, the results were sufficiently encouraging for us to try to localize the site of action of indomethacin. We therefore devised a method aimed at producing a high concentration of indomethacin near the site of formation of luteolysin, namely, the endometrium. For this purpose the indomethacin was mixed with paraffin wax so that it would be slowly released in the neighbourhood of the endometrium over a prolonged period. Although the total amount of indomethacin placed in each uterine horn was large (33 mg), subsequent analysis of the indomethacin content of the implants removed at autopsy indicated that the quantities released in each horn averaged between 0·2 and 0·6 mg/day. This dose was sufficient to produce a considerable increase in the length of the oestrous cycle.

In three animals vaginal smears showed that oestrus had still not occurred up to the 47th day when the animals were killed. In these animals plasma progesterone

levels were high (see Table 3), the normal peak of progesterone levels being about 3 ng/ml, and the corpora lutea were larger than normal resembling those produced by hysterectomy (Rowlands, 1961). In the other three indomethacin-treated guinea-pigs, cycles of 70, 75 and 51 days were observed. These are greatly in excess of normal and are similar to the length of cycles seen in hysterectomized guinea-pigs (Loeb, 1923). When these animals were killed at the middle of a subsequent cycle normal progesterone levels (Challis, Heap & Illingworth, 1971) and corpora lutea of normal size (Bland & Donovan, 1966) were found.

The three control animals killed at the end of the cycle had progesterone levels within the normal range for that time ($<0{\cdot}5$ ng/ml) and the corpora lutea were typically small and had regressed. The three control animals killed at mid-cycle, however, had elevated plasma progesterone levels but the corpora lutea were of normal size. This may be associated with the finding that these guinea-pigs were exhibiting shorter oestrous cycles.

In the control animals, the cycle in which paraffin wax was implanted was significantly prolonged ($P<0{\cdot}001$) and the subsequent cycles were significantly shortened ($P<0{\cdot}001$). These results are identical to those of Ginther (1969) and are due to uterine ligation.

In the control animals, the uteri when homogenized and incubated *in vitro* showed normal variation in prostaglandin production, greater amounts being produced at the 15th day than mid-cycle (Poyser, 1972). There was also normal production in the homogenized uteri from all the animals implanted with indomethacin both in those examined at mid-cycle and in two of the three animals killed on day 47. Evidently the amount of indomethacin in the uterine tissue was insufficient to inhibit prostaglandin production by the uterus *in vitro*.

All uteri contained fluid. In the control animals prostaglandin $F_{2\alpha}$ was detected in this fluid in two out of three animals killed at the end of the cycle. Prostaglandin $F_{2\alpha}$ was also detected in the fluid of one indomethacin-treated animal killed on day 47. In this animal the plasma progesterone level was lower than in the two other animals killed on day 47. It is possible that prostaglandin $F_{2\alpha}$ biosynthesis had been inhibited only partially by the indomethacin implant but this production of prostaglandin $F_{2\alpha}$ was insufficient to cause complete corpora luteal regression.

There is an increase in production of prostaglandin $F_{2\alpha}$ by the normal uterus both *in vivo* and *in vitro* towards the end of the oestrous cycle (Blatchley *et al.*, 1972; Poyser, 1972). It is well established that the uterus exerts a luteolytic influence over the ovary in the guinea-pig and that prostaglandin $F_{2\alpha}$ is luteolytic in this species (Blatchley & Donovan, 1969). The present experiments support the hypothesis that the uterus synthesizes prostaglandin $F_{2\alpha}$ towards the end of the cycle and that this prostaglandin $F_{2\alpha}$ exerts a luteolytic effect on the ovaries thus terminating the life of the corpus luteum. By blocking the synthesis of prostaglandin $F_{2\alpha}$ with indomethacin, we have been able to interfere with this process and thus prolong the length of the oestrous cycle.

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