Silastic implants for delivery of oestradiol to mice

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Silastic implants containing oestradiol were developed for delivering a range of physiological concentrations of oestradiol to mice over long periods. The implants consisted of discrete lengths of Silastic tubing containing oestradiol in arachis oil, with a small reservoir of the oestradiol solution at either end of the implant. Studies showed that the release of oestradiol *in vitro* was proportional to the concentration of steroid within the implant. Implants containing oestradiol at concentrations from 1 to 100 $\mu\text{g ml}^{-1}$ could induce biological responses in ovariectomized mice, ranging from minimal effects on uterine weight and vaginal smears to supraphysiological increases in uterine weight and rapid vaginal cornification. Studies of uterine vascular permeability indicated that significant effects occurred within a few hours of initial placement of the implant. These results suggest that the design of the Silastic implants described in this study provides a useful method for delivering controlled and easily manipulated physiological doses of oestradiol to mice.

Introduction

A well established approach in the study of endocrine control is to ablate the source of the hormone and investigate the effects of treatment with exogenous hormone. This approach has dominated studies of the endocrine control of implantation in rodents and has shown that many uterine responses, including the pattern of cell division, uterine closure, uterine receptivity to blastocysts and other decidual stimuli, depend on a strict regimen of oestradiol and progesterone operating within a strict temporal framework (Finn and Martin, 1972). It is well established, for example, that uterine sensitivity to decidual stimuli can be induced in progesterone-dominated uteri by a narrow range of oestradiol concentrations administered s.c., and that the duration of uterine sensitivity is restricted to a transient temporal 'window' (of implantation). However, it is difficult to make any close comparison between the exogenous hormone regimens used in such studies and the physiological endocrine profiles. A major reason for this is that in many previous studies oestradiol has been administered s.c. using oil as a vehicle. Although oestradiol is readily soluble in the oil, which acts as a reservoir and slows release, the effects of the s.c. injections are relatively short lived, and repeated injections have to be given to produce sustained responses. Largely for experimental convenience, these repeated injections tend to be given on a daily basis. However, such injections produce nonphysiological profiles of oestradiol, with initial high (supra-physiological) concentrations of steroid in plasma which rapidly decline as the steroid is released from the oil (Butcher *et al.*, 1978). Normal physiological concentrations will only be generated during a limited period of this profile.

Although such nonphysiological steroid profiles may be acceptable in the study of endocrine events that are simply dependent on a threshold amount of oestradiol, they are not suitable for studying responses that occur only within a limited range of oestradiol concentrations (e.g. uterine sensitization for implantation and decidualization). A further problem is that although s.c. injections are easy to administer, their effects continue until the steroid runs out; they cannot therefore be used in investigations that require only short-term oestradiol support.

The aims of this study were to develop slow release implants for delivering sustained and controlled physiological concentrations of oestradiol to mice over a strictly defined time course. The implant designs were based on the release characteristics of steroid-filled dimethylpolysiloxane (Silastic; Dow Corning, Midland, MI) tubing (Kincl *et al.*, 1968; Kincl and Rudel, 1971). This polymer has been used in many species for the slow delivery of steroids over long periods (e.g. sheep: Dziuk and Cook, 1966; frogs: Vandorpe and Kuhn, 1989; rats: Martel *et al.*, 1989; Arkaravichien and Kendle, 1990; mice: Huet-Hudson *et al.*, 1990). This paper describes the design and release characteristics of Silastic implants that allow controlled oestradiol delivery to mice in amounts that induce physiological responses.

Materials and Methods

Animals

Female Swiss albino mice (A. Tuck & Son Ltd, Battlesbridge, Essex) were approximately 3 months of age, and weighed 25–35 g. Animals were maintained under constant conditions of light (lights on from 06:00 h to 18:00 h) and temperature ($21 \pm 1^\circ\text{C}$) and fed on a pelleted diet (41B Oxoid) along with tap water *ad libitum*. Ovariectomies were performed under ether anaesthesia at least 2 weeks before the start of each experiment.

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Fig. 1. (a) Log₁₀ of mean daily oestradiol release (ng day⁻¹) from implants ($n = 2$ per group) containing 1 (●), 3 (○), 10 (■), 25 (□) and 100 (▲) µg ml⁻¹ of oestradiol in arachis oil. (b) Hourly release of oestradiol (ng h⁻¹) from implants ($n = 5$ per group) containing oestradiol at a concentration of 10 µg ml⁻¹ arachis oil that had been preincubated (●) in gelatin-phosphate-buffered saline (GPBS) at 37°C for 24 h before use, or used immediately after production (○).

Fig. 2. Vaginal smear response to oestradiol treatment via Silastic implants containing (■) 0 and (□) 1, 3, 10, 20, 50 and 100 µg oestradiol ml⁻¹ arachis oil. Ovariectomized mice were treated with oestradiol from day 1 and vaginal smears were taken on days 4, 6 and 8. Smears were given a score of 1 to 5, representing increasing degrees of cornification. Each point represents the mean $\pm$ SEM for five animals.

Steroid administration

Oestradiol (Sigma Chemical Co., Poole, Dorset) was administered either by s.c. injection in 100 µl arachis oil (BDH Chemicals Ltd, Dagenham, Essex), or in s.c. Silastic implants containing solutions of the appropriate steroid (see below).

Silastic implants

Polythene tubing (Portex Ltd, Hythe, Kent) with an inner diameter of 1.02 mm and an outer diameter of 1.98 mm was inserted 0.5 cm into each end of a 2 cm length of Silastic tubing (inner diameter 1.57 mm, outer diameter 3.18 mm; Dow

Fig. 3. Uterine weights (mg) $\pm$ SEM of ovariectomized animals treated with Silastic implants containing oestradiol at different concentrations for 8 days. *Significantly different from all other groups ($P < 0.0001$).

Corning, Midland, Michigan). This design provided a 1 cm length of the Silastic in the central region of each implant through which steroid release could occur, and a 0.5 cm reservoir of steroid at either end of the implant. The implants were filled by injecting oil containing dissolved oestradiol through the polythene tubing at one end until the entire length of Silastic and polythene tubing was filled. The polythene ends of the implant were heat-sealed with a soldering iron so that they were flush with the ends of the Silastic. After being filled, the implants were washed thoroughly in 90% alcohol followed by distilled water. Implants were normally incubated in gelatine-phosphate-buffered saline (GPBS) at 37°C for 24 h before use. Implants were inserted s.c. into the back of the animal under ether anaesthesia.

Table 1. Uterine vascular permeability (albumin volume; $\mu\text{l mg}^{-1} \times 10^2$) and weights (mg) in ovariectomized female mice treated with oestradiol via different routes of administration

Route of oestradiol treatment	Time from start (h)	<i>n</i>	Uterine weight (mg)	Vascular permeability* ($\mu\text{l mg}^{-1} \times 10^2$)
Controls	0	18	25.00 $\pm$ 0.94	3.64 $\pm$ 0.26
i.v. injection	0.5	8	30.52 $\pm$ 1.74 ^a	2.19 $\pm$ 0.13 ^b
	1	8	31.57 $\pm$ 1.63 ^a	8.58 $\pm$ 1.24 ^a
	2	8	30.95 $\pm$ 1.50 ^a	10.81 $\pm$ 2.04 ^a
	4	8	32.67 $\pm$ 2.40 ^a	12.53 $\pm$ 2.06 ^a
s.c. injection	2	8	24.49 $\pm$ 1.20	4.85 $\pm$ 0.70
	4	8	30.77 $\pm$ 1.60 ^{ab}	15.24 $\pm$ 2.51 ^{ab}
s.c. implant (not preincubated)	2	8	23.79 $\pm$ 0.58	3.55 $\pm$ 0.40
	4	8	23.90 $\pm$ 2.00	13.98 $\pm$ 2.02 ^{ab}
s.c. implant (preincubated)	4	8	26.76 $\pm$ 2.30	5.32 $\pm$ 1.21 ^a
	6	8	26.50 $\pm$ 1.08	6.30 $\pm$ 1.20 ^a
	8	8	31.74 $\pm$ 1.00 ^a	6.10 $\pm$ 0.14 ^a
	12	8	56.43 $\pm$ 2.33 ^{ab}	30.05 $\pm$ 3.24 ^{ab}

Steroid treatment commenced at 0 h and vascular responses were assessed at various times afterwards. *Vascular permeability was assessed by the uterine extravascular accumulation of ^{125}I -labelled albumin.

^aStatistically significant differences ($P < 0.05$) from control values and ^bstatistically significant difference from other groups treated via the same route ($P < 0.05$).

Radioactively labelled steroids

62.5 μCi [2,4,6,7- ^3H]oestradiol (Amersham Int., London) was mixed with unlabelled oestradiol at a concentration of 1 mg ml^{-1} to produce a solution in arachis oil with a specific activity of 5666.7 $\mu\text{Ci mmol}^{-1}$. Appropriate dilutions were made in arachis oil to give final concentrations of 1, 3, 10, 25, 100 and 1000 $\mu\text{g ml}^{-1}$.

Experiment 1: long-term release of oestradiol from implants in vitro

Two implants were filled with each of the steroid concentrations containing radioactively labelled tracer. The implants were preincubated for 24 h before use. Individual implants were placed in 4 ml of GPBS at 37°C and the medium was changed every 24 h for 35 days. At various times over this period, the radioactivity released into the GPBS over 24 h was determined after mixing with 11 ml Cocktail T Scintillant (Amersham Int., London), using a Packard 460C Scintillation Counter (Canberra Packard, Pangbourne, Berkshire). The rate of steroid release from the implant was determined from the specific activity of each solution.

Experiment 2: initial release rates of oestradiol from implants

Implants containing mixtures of labelled and unlabelled oestradiol at concentrations of 10, 25, 100 and 1000 $\mu\text{g ml}^{-1}$ were used to investigate hourly release rates. The implants (five of each concentration) were either preincubated for 24 h or used

soon after production. Each implant was incubated in 4 ml GPBS at 37°C for 1–3 h before being placed in fresh GPBS. The rate of release of oestradiol in each incubation period was calculated as above. Release rates were monitored for a period of 53 h.

Experiment 3: uterine weight and vaginal smear responses to oestradiol-containing Silastic implants

Two established, sensitive bioassays (vaginal smears and uterine weight) were used to monitor the chronic *in vivo* effects of the oestradiol-containing implants. Silastic implants containing oestradiol at concentrations of 1, 3, 10, 50 or 100 $\mu\text{g ml}^{-1}$ arachis oil were placed s.c. into ovariectomized female mice ($n = 5$ per group). Control females received empty implants. Vaginal smears were taken on days 4, 6 and 8, and scored on an arbitrary scale of 1–5 based on the proportion of cornified epithelial cells (1 = no cornified cells; 5 = fully cornified) in the smear. The mice were killed on day 8 and the uteri were weighed.

Experiment 4: vascular permeability changes in response to oestradiol treatment via different routes of administration

The ability of oestradiol to induce a rapid increase in uterine vascular permeability in ovariectomized mice was used to investigate acute responses to the placement of oestradiol implants. Vascular permeability was assessed using the method described by Arvidson (1977). In brief, mice were subjected to ether

anaesthesia followed by i.v. injection of 0.5 μCi [^{125}I]-labelled human serum albumin (HSA). Thirty minutes later a blood sample was obtained by cardiac puncture and the animal was killed by cervical dislocation under light ether anaesthesia. Both uterine horns were removed, cleared of all fat and mesentery, weighed, rinsed in saline and blotted dry. The radioactivity in the uterus and in a 100 μl plasma sample was determined. The tissue-specific albumin volume (AV) was expressed as a ratio of the c.p.m. mg^{-1} tissue to c.p.m. μl^{-1} plasma. The AV value is a good index of tissue vascular permeability (Arvidson, 1977).

Ovariectomized females were treated with oestradiol in one of the following ways: (i) i.v. injection of 100 ng oestradiol in 0.1 ml 0.9% saline; (ii) 100 ng oestradiol s.c. injection in 0.1 ml arachis oil; (c) s.c. placement of a Silastic implant containing 50 μg oestradiol ml^{-1} solution in arachis oil. The implants were used either immediately after production or were incubated in GPBS at 37°C for 24 h before use. Uterine weights and vascular permeability were assessed at various times (ranging from 0.5 to 12 h) after injection or implant placement.

Statistical analysis

All results were expressed as means $\pm$ SEM. Analysis of variance (ANOVA) and Gabriel's test for multiple comparisons were performed as necessary.

Results

Experiment 1: long-term release from oestradiol containing implants in vitro

Rates of daily release of oestradiol from these implants declined steadily over the sampling period (Fig. 1a). The amount of steroid released per day was proportional to the concentration of steroid within the implants (linear correlation, $r = 0.997$).

Experiment 2: initial rates of release of oestradiol from implants

Steady rates of oestradiol release were obtained from implants that had been preincubated in GPBS before use (Fig. 1b) and the release rates showed a linear relationship with the concentrations within the implant. The mean hourly release rates ($\pm$ SEM) for preincubated implants containing oestradiol at 10, 25, 100 and 1000 $\mu\text{g ml}^{-1}$ were 0.4 ± 0.1 , 1.1 ± 0.1 , 5.7 ± 0.6 and 62.3 ± 3.2 ng h^{-1} , respectively. In contrast, oestradiol release from implants that had not been incubated for 24 h before use were considerably higher for the first few hours of the experimental sampling period, but declined very rapidly to reach values similar to those obtained from the preincubated implants (Fig. 1b).

Experiment 3: uterine weight and vaginal smear responses to oestradiol-containing Silastic implants

Implants containing high concentrations of oestradiol induced more rapid changes in the vagina than did lower doses of steroid. Changes in the vaginal smear occurred at doses of

oestradiol as low as 3 $\mu\text{g ml}^{-1}$ and full vaginal cornification could be obtained within 8 days with implants containing 10 $\mu\text{g ml}^{-1}$ or more of oestradiol (Fig. 2). Furthermore, implants containing oestradiol at concentrations of 10 $\mu\text{g ml}^{-1}$ and above caused significant increases in uterine weight (Fig. 3). While uterine weights in animals treated with the highest doses of oestradiol reached over 200 mg, this represents a supraphysiological response: uterine weights in early pregnancy, for example, are typically only about 50–70 mg.

Experiment 4: vascular permeability changes in response to oestradiol treatment via different routes of administration

The earliest uterine vascular permeability responses occurred in animals treated with oestradiol via the i.v. route, and significant increases occurred within 1 h of treatment (Table 1). Animals treated with s.c. injections or with unincubated implants showed no significant effects at 2 h, but uterine vascular permeability was increased by 4 h after the initial application of the steroid ($P < 0.05$). Mice treated with preincubated implants showed a small rise in vascular permeability at 4 and 8 h after implant placement, rising to a tenfold increase in albumin volume by 12 h.

Small, but significant, increases in uterine weight were observed in the i.v. and s.c. injection groups, within 0.5 and 4 h respectively, after oestradiol treatment (Table 1). No significant change in uterine weight occurred during the 4 h experiment in animals treated with nonincubated implants but significant uterine weight increases were observed after 8 h of treatment with preincubated implants ($P < 0.05$).

Discussion

The results of these studies indicate that slow-release, oestradiol-containing Silastic implants, suitable for use in studies of physiological responses in mice, can be made and used with relative ease. This should allow such implants to be readily integrated into established endocrine regimens. The implants combine the advantages of controlled delivery of physiological amounts of oestradiol over long periods with the ability to produce rapid changes by removing the implant and replacing it with one having different release characteristics.

Although previous workers have used various designs of Silastic implant for the study of endocrine responses in small rodents, few studies have been undertaken to produce implants that can deliver oestradiol at physiological concentrations. Bridges (1984) showed that small (1–2 mm) Silastic implants containing crystalline oestradiol generated plasma concentrations of oestradiol similar to those found in intact rats. Huet-Hudson *et al.* (1990) used Silastic implants filled with crystalline oestradiol to investigate synthesis of uterine epidermal growth factor (EGF) in ovariectomized female mice. However, the use of crystalline oestradiol in the production of implants can present difficult handling problems with dangers of contamination. In addition, although plasma oestradiol provides a direct measure of the oestradiol concentrations attained, this method of assessment is only possible when the concentrations of oestradiol are relatively high. At low physiological concentrations,

biological responses may provide a better method for assessing the amount of steroid delivered by the implant. In the study reported here, the use of two particularly sensitive biological responses (vaginal smears and uterine weight) allowed the identification of a suitable range of oestradiol concentrations within implants for the delivery of physiological amounts of oestradiol: although implants containing oestradiol at a concentration of $1\text{--}3\text{ }\mu\text{g ml}^{-1}$ produced only very small uterine or vaginal responses, full responses could be obtained by concentrations above about $20\text{ }\mu\text{g ml}^{-1}$.

The *in vitro* studies confirmed the importance of preincubating the implants *in vitro* before s.c. insertion to obtain steady-state release rates: the initial release of steroid from implants that were not treated in this way was far higher than from implants that were preincubated, reflecting the rapid loss of the oestradiol that had accumulated within the Silastic itself. The rapid release of oestradiol from such implants was reflected by responses *in vivo*: the implants that had not been preincubated produced effects on vascular permeability within 4 h, similar to the responses to oestradiol administered s.c. in oil. In contrast to these rapid responses, the effects of implants that had been preincubated in GPBS before insertion were somewhat slower, with only small responses occurring within the first 8 h of implant placement. Huet-Hudson *et al.* (1990) observed very high concentrations of oestradiol 12 h after implant insertion, with a subsequent rapid decline in plasma oestradiol: this presumably also reflected the initial rapid loss of oestradiol accumulated within the Silastic.

The amount and duration of oestradiol release from the implants depends on several variables, including implant dimensions and internal steroid concentration. In the present study, and after initial preliminary experiments, implant dimensions were standardized and only the internal oestradiol concentration was changed. Oestradiol release was relatively stable on a hour to hour basis, but declined gradually over the 35 days. This slow decline reflected the progressive loss of oestradiol from the oil within the implant, and a consequent progressive decline in the internal oestradiol concentration. It is therefore recommended that, in studies involving the use of these implants for more than just a few days, either the length

of the oil reservoirs at either end of the implant be increased or the implants be replaced on a regular basis (e.g. every 2 weeks). Despite this limitation, the ease with which these implants can be placed, removed, or substituted with implants containing different internal steroid concentration provides a useful method for delivering a range of controlled, physiological concentrations of oestradiol to mice.

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