Effects of an LH-RH analogue in male rats pretreated with oestradiol benzoate

R. K. Tcholakian, A. de la Cruz, A. K. Chowdhury, Mridula Chowdhury, A. V. Schally* and Anna Steinberger

*Department of Reproductive Medicine and Biology, The University of Texas Medical School at Houston, Texas 77030 and the *Endocrine and Polypeptide Laboratories, Veterans Administration Hospital, New Orleans, Louisiana 70140, U.S.A.*

Summary. Treatment of adult male rats with oestradiol benzoate (OB) for 21 days significantly decreased the body, testicular and accessory sex organ weights but increased anterior pituitary weight. OB treatment also significantly suppressed circulating FSH and LH levels as well as plasma and testicular concentrations of testosterone. The seminiferous tubules and interstitial cells were partly atrophied, and there was some effect on spermatogenesis, with step 14 to 19 spermatids being fewer than normal.

Rats treated with OB for 21 days were then treated daily with LH-RH analogue ({D-Leu⁶, des-Gly-NH₂¹⁰}-LH-RH-ethylamide), to see if testicular function could be recovered. Circulating gonadotrophins were significantly elevated, testicular histology was normal and testicular and plasma testosterone concentrations and the accessory sex organ weights remained suppressed. These results suggest possible extra-pituitary effects of the LH-RH analogue, including a direct action on the testes and/or accessory sex organs.

Introduction

The effects of high doses of oestradiol benzoate administered to intact adult male rats for 1 day (Tcholakian, Chowdhury & Steinberger, 1974), or daily for 28 (Chowdhury, Tcholakian & Steinberger, 1974) or 40 (Kincl *et al.*, 1965) days have been described. Tcholakian *et al.* (1974) demonstrated that a single subcutaneous (s.c.) injection of 50 µg inhibited testosterone production within a few hours and that about 6 days were required before the testes recovered their normal steroidogenic function (Tcholakian, Chowdhury & Chowdhury, 1978). Chronic daily treatment for 2 weeks produced atrophy of the testes and accessory sex organs (Chowdhury & Steinberger, 1975; Tcholakian *et al.*, 1978), decreased plasma and testicular testosterone concentrations and significantly reduced pituitary LH concentration. These suppressive effects on the testis and pituitary persisted for 60 days after cessation of treatment. One attempt to speed the recovery of oestrogen-‘damaged’ testes by acute treatment with exogenous LH indicated that, although LH (1–100 µg/rat) significantly elevated serum testosterone levels in oestrogen-treated and control rats, those in the treated animals were significantly lower than in the control animals (Moger, 1976).

The recent availability of an analogue of LH-RH which has intense and prolonged gonadotrophin-releasing activities in the rat *in vivo* (Vilchez-Martinez *et al.*, 1974; de la Cruz *et al.*, 1975) and *in vitro* (Coy, Labrie, Savary, Coy & Schally, 1976) has led us to investigate its ability to speed the recovery of testicular function in rats treated with oestradiol benzoate.

Materials and Methods

The studies were performed on 90-day-old Long-Evans rats, raised in our colony in a controlled temperature (21 ± 1 °C) and light (12 h light/24 h) environment. The animals were allowed free access to food and water throughout the experiment.

Experiments

In Exp. I, a group of 5 animals received daily subcutaneous injections of 50 µg oestradiol benzoate (1,3,5(10)-estratrien-3,17β-diol 3-benzoate: E.970-Steraloids, Inc., Wilton, New Hampshire) in 0.1 ml sesame oil for 21 days. A control group of 5 animals was injected with the same volume of the oil vehicle alone. In Exp. II, 5 groups of 5 animals were pretreated with 50 µg oestradiol benzoate in 0.1 ml sesame oil for 21 days. Group B was rested for the next 30 days; Group C was treated with diluent alone for 30 days and Groups D, E and F were each treated daily for 30 days with LH-RH analogue ($\{D\text{-Leu}^6, \text{des-Gly-NH}_2^{10}\}$ -LH-RH-ethylamide: supplied by courtesy of Dr A. V. Schally, Endocrine and Polypeptide Laboratories, Veterans Administration Hospital, New Orleans, Louisiana, U.S.A.). The LH-RH analogue was dissolved in diluent (0.1 % bovine serum albumin: 0.9 % sodium chloride) and administered in 0.1 ml volumes twice daily at 09:00 and 18:00 h for a total daily dose of 2 (Group F), 20 (Group E) or 200 (Group D) ng per animal. The 5 rats in Group A were untreated.

Body weights were recorded before and at the end of each treatment, and the animals were killed 1 day after the last injection. Individual blood samples were collected into heparinized syringes by cardiac puncture while the animals were under ether anaesthesia. Serum and plasma were frozen and stored at -20°C for subsequent assay of gonadotrophins and testosterone, respectively. The anterior pituitary, accessory sex organs and testes were removed and weighed. One testis from each animal was fixed in Bouin's fluid and stained with PAS-Weigert haematoxylin for histological analysis. The other testis was frozen in 2 ml saline (0.15 M-NaCl) and stored at -20°C for subsequent testosterone determinations.

Assays

Gonadotrophins. Serum LH and FSH were measured by a double-antibody radioimmunoassay with reagents provided by the National Pituitary Agency as described elsewhere (Chowdhury & Steinberger, 1976). Rat-FSH-RP1 ($2.1 \times \text{NIH-FSH-S1}$) and rat-LH-RP1 ($0.03 \times \text{NIH-LH-S1}$) were used as reference standards. The minimum sensitivity of the assay was 12.5 ng/tube for FSH and 3 ng/tube for LH, with a mean intra-assay variation of 4.5 % for FSH and 3.3 % for LH. All determinations were performed in a single assay, 1 point per sample of 0.1 ml volume with a correlation coefficient of -0.996 for LH and -0.986 for FSH.

Testosterone. A radioligand immunoassay was used for determination of plasma and testicular concentrations (Tcholakian *et al.*, 1978). The antibody was raised in rabbits against a 19-carboxymethyloxime ether derivative of testosterone conjugated to bovine serum albumin (Rao, Moore, Peterson & Tcholakian, 1978). The antiserum cross-reacted with other steroids as follows: testosterone, 100 %; 5α-dihydrotestosterone, 6.65 %; 5α-androstane-3α,17β-diol, 2.2 %; all other steroids, 0–<1 %. All samples were measured in a single assay with an intra-assay variation of 6 % and a sensitivity of 0.2 ng/ml plasma.

Statistical analysis

The computations and statistical analysis of the data were performed with a Hewlett-Packard 9830 programmable calculator according to the Fortran Computer Program of Rodbard & Lewald (1970).

Results

Body and organ weights

Treatment with oestradiol benzoate for 21 days significantly decreased body and accessory sex organ weights, but increased the weight of the anterior pituitary when compared to the controls (Table 1). The lower doses (2 or 20 ng/day, Groups F and E) of the analogue produced no significant changes. Comparison of untreated animals (Group A), with oestradiol benzoate-treated (Groups B and C) and analogue-treated (Group D) animals (Table 2) showed that the significant body weight loss after oestradiol benzoate treatment remained suppressed for 30 days after cessation of the treatment, while no significant difference was observed between the anterior pituitary and testicular

weights in the groups. Furthermore, comparison of Group C with Group D (Table 2) showed no change in body, anterior pituitary or testicular weights, although the weights of the accessory sex organs were significantly lower in Group D.

Table 1. Experiment I: the effects (mean $\pm$ s.e.m.) of treatment with oestradiol benzoate (50 μ g/day for 21 days) on adult male rats (5/group)

	Vehicle only	Oestradiol benzoate
Body wt (g)	302.4 $\pm$ 5.4	267.2 $\pm$ 10.2 ^a
Wt of anterior pituitary (mg)	8.2 $\pm$ 0.3	18.9 $\pm$ 1.7 ^b
Wt of testes (g)	2.45 $\pm$ 0.95	1.97 $\pm$ 0.46
Wt of ventral prostate (mg)	227.0 $\pm$ 19.4	47.2 $\pm$ 4.0 ^c
Wt of seminal vesicles (full) (mg)	932.4 $\pm$ 41.0	142.4 $\pm$ 11.9 ^c
Serum LH (ng/ml)	47.3 $\pm$ 4.7	11.4 $\pm$ 1.2 ^c
Serum FSH (ng/ml)	273.5 $\pm$ 41.3	144.0 $\pm$ 14.7 ^a
Plasma testosterone (ng/100 ml)	141.7 $\pm$ 11.9	19.9 $\pm$ 2.3 ^c
Testicular testosterone (ng/g)	52.4 $\pm$ 4.0	8.4 $\pm$ 0.1 ^c

Superscript letters indicate values significantly different from those of the vehicle-injected rats: ^a $P < 0.05$, ^b $P < 0.005$, ^c $P < 0.001$ (Student's *t* test).

Table 2. Experiment II: the effects (mean $\pm$ s.e.m.) of treatment with oestradiol benzoate (OB: 50 μ g/day for 21 days) followed by treatment with an LH-RH analogue (200 ng/day for 30 days) on adult male rats (5/group)

	Group A (no treatment)	Group B (OB + no treatment for 30 days)	Group C (OB + diluent for 30 days)	Group D (OB + LH-RH ana- logue for 30 days)
Body wt (g)	372.1 $\pm$ 13.9	319.0 $\pm$ 11.3 ^a	331.8 $\pm$ 6.0 ^a	319.2 $\pm$ 6.2 ^a
Wt of anterior pituitary (mg)	7.7 $\pm$ 0.3	10.1 $\pm$ 1.2	10.2 $\pm$ 1.0	10.5 $\pm$ 0.4
Wt of testes (g)	2.94 $\pm$ 0.5	2.43 $\pm$ 0.6	2.46 $\pm$ 0.57	2.38 $\pm$ 0.64
Wt of ventral prostate (mg)	379.4 $\pm$ 24.2	319.0 $\pm$ 19.0	270.4 $\pm$ 22.5 ^a	170.3 $\pm$ 7.6 ^{a*}
Wt of seminal vesicles (full) (mg)	1082.6 $\pm$ 88.0	942.0 $\pm$ 49.0	977.6 $\pm$ 89.6	195.4 $\pm$ 4.1 ^{†‡}
Serum LH (ng/ml)	33.8 $\pm$ 3.6	18.7 $\pm$ 4.2 ^a	15.2 $\pm$ 4.1 ^a	50.3 $\pm$ 4.8 ^{a†}
Serum FSH (ng/ml)	289.5 $\pm$ 12.8	364.8 $\pm$ 33.1	245.5 $\pm$ 11.0 ^{a§}	367.4 $\pm$ 14.4 ^{a†}
Plasma testosterone (ng/100 ml)	140.0 $\pm$ 19.6	296.8 $\pm$ 62.3	189.0 $\pm$ 20.7	51.7 $\pm$ 5.5 ^{b†}
Testicular testosterone (ng/g)	93.6 $\pm$ 11.8	91.0 $\pm$ 16.2	91.8 $\pm$ 8.0	7.9 $\pm$ 1.8 ^{a*}

Values are significantly different from those in Group A: ^a $P < 0.05$, ^b $P < 0.01$, ^c $P < 0.001$ (Student's *t* test).

Values are significantly different from those in Group C: ^a $P < 0.01$, [†] $P < 0.005$, [‡] $P < 0.001$ (Student's *t* test).

§ Value significantly different from that in Group B, $P < 0.05$ (Student's *t* test).

Testicular histology

Treatment of adult rats with oestradiol benzoate for 21 days led to partial atrophy of the seminiferous tubules and the interstitial cells. The tubular cross-sections appeared smaller, and in some tubules step 14 to 19 spermatids were fewer than normal. Cell associations in other stages appeared normal. Daily injections of the oestradiol-treated animals with 200 ng LH-RH analogue for 30 days (Group D) improved spermatogenesis and it was indistinguishable from that in animals treated only with diluent (Group C).

Serum gonadotrophin concentrations

LH and FSH levels were significantly lower in the oestradiol-treated animals than in the controls (Table 1). Subsequent treatment with the LH-RH analogue produced significant increases ($P < 0.005$) in both serum gonadotrophins in Group D animals compared with those in Group C rats (Table 2).

Plasma and testicular testosterone concentrations

Plasma testosterone levels in the Group D rats were significantly suppressed ($P < 0.005$) when compared to those in rats in Groups B or C (Table 2), but this suppression was not as marked as that observed after oestradiol benzoate treatment (Table 1). Testicular testosterone concentrations were significantly decreased in the oestradiol-treated rats in Exp. I (Table 1) and in the analogue-injected animals in Exp. II (Table 2). Values for rats in Groups B and C were not significantly different from those of normal untreated rats in Group A (Table 2).

Discussion

The effects in male rats of daily administration of oestradiol benzoate for 21 days on body weight, pituitary, testicular and accessory sex organ weights and on serum gonadotrophins and plasma and testicular concentrations of testosterone in this study agree with previous reports on its effects in adult male rats treated daily for 14 days (Tcholakian *et al.*, 1978), 28 days (Chowdhury *et al.*, 1974) or 40 days (Kincl *et al.*, 1965). The suppressive effect of oestradiol benzoate on the gonado-pituitary axis appears to be due to (1) the direct effect of oestrogens on the testes, possibly via suppression of steroid enzymes concerned with testosterone synthesis (Samuels, Short & Huseby, 1964; Oshima, Wakabayashi & Tamaoki, 1967; Moger, 1976; Kremers, Tixhon & Grielen, 1977), and (2) the suppression of pituitary gonadotrophins which regulate androgen synthesis (Bogdanove, Diebel, Story & Kingsley, 1971; Tcholakian *et al.*, 1978).

The recovery patterns of testosterone and gonadotrophins seem to indicate that oestradiol benzoate may act independently on the testes and the pituitary because the testes recover to control levels earlier than does the pituitary (Tcholakian *et al.*, 1978). In the present study most of the suppressive effects of oestradiol benzoate were diminished by 30 days after the end of treatment except that serum LH levels remained significantly low. The significant ($P < 0.005$) elevation in the circulating levels of both gonadotrophins caused by 200 ng [D-Leu⁶, des-Gly-NH₂¹⁰]-LH-RH-ethylamide is a direct activity of the analogue that overcame the suppressive effects of oestradiol benzoate, indicating a strong gonadotrophin-releasing activity of this analogue in male rats. A single dose of 50 ng analogue maintained elevated serum LH and FSH levels for up to 6 h after administration (Vilchez-Martinez, Pedroza, Coy, Arimura & Schally, 1977). Although treatment with 200 ng LH-RH analogue overcame the suppressive effects of oestradiol treatment on serum gonadotrophin concentrations, and on spermatogenesis, the weights of the accessory sex organs and testicular and plasma concentrations of testosterone were not restored to control values. The significant suppression of accessory sex organ weights existing even after chronic treatment with the LH-RH analogue and elevation of serum gonadotrophin levels is compatible with the observed significant decreases in testicular and plasma testosterone concentrations. The increase in circulating LH during the recovery period after chronic treatment with oestradiol benzoate in male rats does not necessarily coincide with increases in testosterone production (Tcholakian *et al.*, 1978). It has been suggested that chronic treatment with oestradiol benzoate may produce a long-term effect on the ability of the testes to respond to endogenous or exogenous LH (Samuels *et al.*, 1964; Tcholakian *et al.*, 1978). Our present data support this suggestion.

It is probable that the LH-RH analogue has a direct inhibitory effect on testicular steroidogenesis in addition to stimulating gonadotrophin secretion. Recent observations that synthetic LH-RH and its superactive analogues may have a direct suppressive action on the ovary and uterus of rats (Johnson, Gendrick & White, 1976; Rippel & Johnson, 1976; Humphries, Wan, Folkers & Bowers, 1976; Corbin, Beattie, Yardley & Foell, 1976) add further support to the possibility of there being a direct action of this analogue on the testis. The observations of Auclair, Kelly, Coy, Schally & Labrie

(1977) suggest that such a direct action of the analogue on the testis involves significant reduction (80%) of testicular LH/hCG receptor levels and this could account for the fact that increased circulating gonadotrophins failed to stimulate testicular steroidogenesis. Further studies are needed to clarify the extra-pituitary effects of LH-RH and its analogues.

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