Sustained Suppression of the Pituitary-Gonadal Axis by Leuprorelin Three-Month Depot Microspheres in Rats and Dogs[1]

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The pharmacological effects of leuprorelin three-month depot microspheres were investigated in rats and dogs. After s.c. and i.m. injection, the microspheres provided similar linear drug release and sustained serum drug levels for 3 months. Persistent suppression of serum LH, FSH (in rats) and testosterone (in rats and dogs) for over 16 weeks was achieved when the microspheres were given at a dose of 100 (rat) and 25.6 (dog) μg/kg/day. These hormone release responses upon periodic challenge tests revealed that a single injection of the microspheres caused dramatic suppression of the function of the pituitary-gonadal system for 15 weeks in rats. The growth of the genital organs was also suppressed dose-dependently by injection of the microspheres over 3 months; the strongest suppression was achieved at a dose of 100 μg/kg/day. This three-month depot formulation is expected to be more convenient than the one-month depot with improved patient compliance and therapeutic effects.

KEY WORDS: leuprorelin (leuprolide), three-month depot microspheres, sustained drug release, pharmacological effects, suppression of pituitary-gonadal axis, rats and dogs.

INTRODUCTION

Three-month release injectable microspheres of leuprorelin acetate (leuprolide acetate, D-Leu^6-(des-Gly^{10}-NH_2)-LH-RH ethylamide) have been developed in order to attain a more convenient and reliable dosage formulation for hormonal therapy of prostate cancer and endometriosis (1,2). In our previous study (2), the formulation and preparation procedures for the three-month release microspheres of leuprorelin acetate were examined using an *in vivo* release test using 50 different preparations of poly(DL-lactic acid) (PLA) and poly(DL-lactic/glycolic acid) (PLGA). Microspheres prepared by the in-water drying method and using PLA with a molecular weight of 12,000-18,000 were found to provide linear sustained release and persistent plateau serum levels of the drug for over 13 weeks in rats after s.c. injection. Furthermore, the microspheres prepared using PLA with a m.w. of about 15,000, containing less than 0.1% of water-soluble oligomers and loaded with 12% of the drug were considered optimal for the injectable three-month depot formulation.

One-month release injectable PLGA microspheres of three agonistic LH-RH analogs [leuprorelin (in-water drying method) (3,4), nafarelin (phase separation method) (5,6) and triptorelin (phase separation method) (7)] have been reported, but only our one-month depot product (Lupron) is currently registered in the United States, and no one has of yet successfully developed a three-month depot injection of any of these analogs.

In the present study, we examined the drug remaining at the injection site and drug serum levels, gonadotropin and testosterone in rats and dogs after s.c. and i.m. injection of leuprorelin three-month depot microspheres prepared with PLA of m.w. of 15,500 and 15,800. The function of the pituitary-gonadal system with regard to LH, FSH and testosterone release was determined by a challenge test in rats after a single injection of the microspheres. Furthermore, weight changes in the genital organs were examined in rats after injection of the microspheres at four different doses.

MATERIALS AND METHODS

Animals and Materials

Male Sprague-Dawley rats (10 weeks of age) and beagle dogs (7 months of age) were purchased from Clea Japan, Inc. (Tokyo) and Toyo Research Animals, Inc. (Shizuoka), respectively. Leuprorelin acetate was synthesized at Takeda Chem. Ind., Ltd. (Lot M548-140) and Abbott Labs. (North Chicago, IL) (Lot 37-780-CE). PLA was purchased from Wako Pure Chem. Ind., Ltd. (Tokyo). The microspheres of leuprorelin acetate with PLA (PLA-15,800 for Lot 0603912 and PLA-15,500 for Lot 403 and 061390) were prepared as previously reported (2). Briefly, in the case of Lot 061390 of the microspheres, 5.4 g of the drug (Lot 37-780-CE) dissolved in 5 ml of distilled water and PLA (40 g) dissolved in 45 ml of CH_2Cl_2 were mixed and agitated vigorously with a homogenizer. This emulsion was added to 10 L of 0.1% polyvinyl alcohol solution (about 18°C) under stirring with a mixer. The resulting (w/o)/w emulsion was stirred gently for 3 hr to remove the organic solvent. The microspheres were washed with water and lyophilized. Lot 403 of the microspheres was prepared on a scale one-tenth this size using the same polymer. The microspheres were observed under a scanning electron microscope (JSM-T-300, JEOL Ltd. Tokyo).

Radioimmunoassay for Drug and Hormone Levels

Serum levels of leuprorelin, LH, FSH and testosterone were determined in duplicate by double-antibody RIA systems. An RIA system using the chloramine-T radioiodination method was utilized to determine serum leuprorelin as previously reported (4). RIA kits were purchased from Amersham International plc. (Amersham, UK) for rLH and rFSH and from CIS Diagnostics, Ltd. (Chiba) for testosterone. Standard rLH and rFSH in these RIA kits have been calibrated against NIADDK-rLH RP-2 and NIADDK-rFSH RP-2, respectively. Serum testosterone in rats was assayed directly using diluted serum, but in dogs it was determined after being extracted from the serum (50 μl) with ether (3 ml) to eliminate the interference of serum components. The mea-

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surable limit per 1 ml of serum is 63 pg for leuprorelin acetate, 0.2 ng for LH, 0.4 ng for FSH and 31 pg for testosterone.

Drug Release *in Vivo* and Serum Drug and Hormone Levels in Rats

The microspheres (Lot 0603912) were injected s.c. into the back or i.m. into the femoral muscle of rats at a dose of 4.5 mg of the drug in 0.5 ml of vehicle corresponding to a dose of about 100 μg/kg/day of the drug for 90 days (average body weight of 500 g at 16 weeks of age). To determine the amount of drug remaining in the microspheres at the injection site, the microspheres were excised periodically, and residual drug content was assayed by HPLC as described previously (4). Blood was collected periodically from the abdominal aorta under ether anesthesia, and the serum was stored at −40°C until the day of RIA for the drug and hormone levels.

Serum Drug and Testosterone Levels in Dogs

The microspheres were injected s.c. (Lot 0603912) and i.m. (Lot 061390) into the hind leg of dogs at a dose of 25.6 μg/kg (body weight at the start of treatment)/day of the drug (18.5 mg of drug in 1.5 ml of vehicle for a dog weighing 8 kg). Blood (about 2 ml) was withdrawn periodically from the forearm vein, and the serum was stored at −40°C until determination of the drug and testosterone levels.

Challenge Test for Hormone Release Responses

To evaluate the duration of suppression of the pituitary-gonadal system, leuprorelin acetate solution (100 μg/kg) was injected s.c. into rats at various times after the microspheres (Lot 061390) had been injected i.m. (seven groups of five rats each at a dose of 4.5 mg of the drug). Blood was collected from a tail vein periodically for 8 hrs after the drug solution was injected for determination of serum LH, FSH and testosterone levels by RIA.

Dose-Dependency of Serum Testosterone and Genital Organ Weight Changes

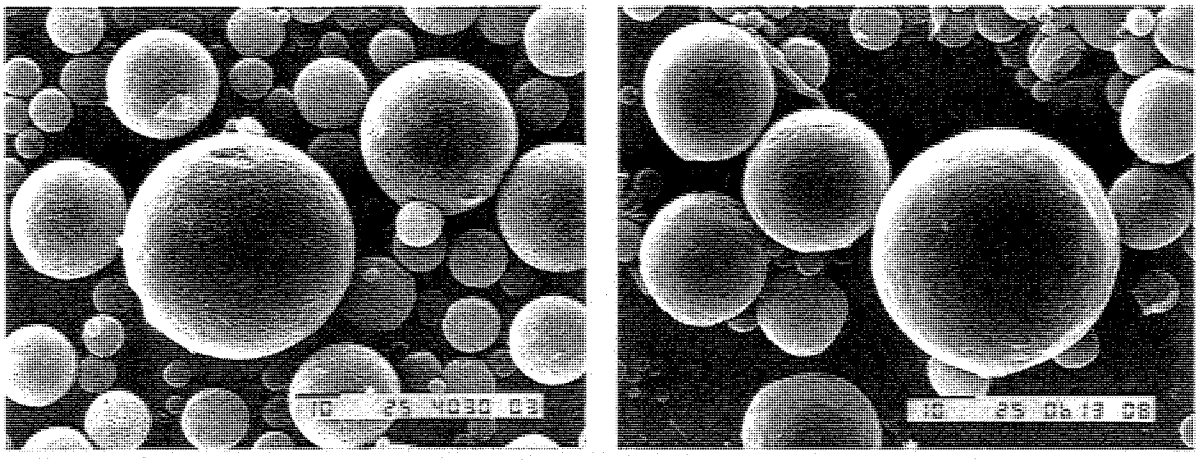
The microspheres (Lot 061390) were injected i.m. into rats at a dose of 0.045, 0.45, 1.35 and 4.5 mg of the drug, corresponding to approximately 1, 10, 30 and 100 μg/kg/day for 90 days, respectively, and the right testis, both seminal vesicles and the prostate glands except for the coagulating glands were carefully removed [seven groups of five rats at each dose]. Each organ was weighed, and the weights were compared with those in untreated rats of the same age bred under the same conditions. To determine the serum testosterone, the microspheres (Lot 061390) were injected i.m. into rats at a dose of 0.45, 1.35 and 4.5 mg of the drug, and blood was collected periodically from a tail vein.

RESULTS AND DISCUSSION

Drug Release *in Vivo*

The pharmacologic activities of leuprorelin three-month depot microspheres prepared with PLA having a m.w. of about 15,500 were investigated in rats and dogs. Fig. 1 shows scanning electron micrographs of the microspheres prepared on a small laboratory scale (left) and a 10-times larger scale (right) by the in-water drying method. These microspheres were fairly spherical particles with a mean diameter of 20–30 μm which is similar to that of the one-month depot formulation.

The leuprorelin release profiles, as % of the dose remaining at the injection site, in rats after s.c. and i.m. injection of the microspheres (Lot 0603912) are shown in Fig. 2. The microspheres provided a relatively linear and well-sustained release of the drug for over 3 months (13 weeks) following both s.c. and i.m. injection. One-day release from the microspheres after s.c. and i.m. injection was 7.7% and 15.8%, respectively. The % remaining at 13 weeks was similar with s.c. and i.m. dosing, and it was less than 10%, providing the desired release profiles. Although i.m. injection resulted in a slightly larger initial burst within the first



Lot 403 Lot 061390

Fig. 1 Scanning electron micrographs of leuprorelin three-month release microspheres. The bar at the bottom in the photographs represents 10 μm.

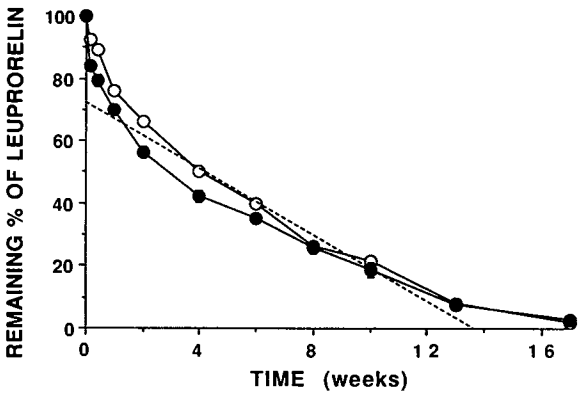


Fig. 2 *In vivo* release of leuprorelin from the three-month depot in rats after s.c. (○) and i.m. (●) injection. (Dose = 4.5 mg/rat, mean±SE, n = 5, regression line: $Y = -0.75X + 71.6$, $r = -0.97$)

day than s.c. injection, possibly because of faster removal from the lymphatic drainage or acute uptake of the fine microspheres by macrophages, the two injection routes provided similar long-term sustained release. The regression line of the remaining amount for one to 13 weeks following injection by either route calculated by least-squares regression analysis indicated that the average drug release rate was 0.75% of the dose/day; the correlation coefficient was 0.97.

Serum Leuprorelin in Rats and Dogs

Serum levels of leuprorelin acetate in rats and dogs after s.c. and i.m. injection of the microspheres are shown in Fig. 3. The serum levels in both animals were elevated just after injection due to the initial burst from the microspheres, but this followed by a persistent plateau level for over 13 weeks.

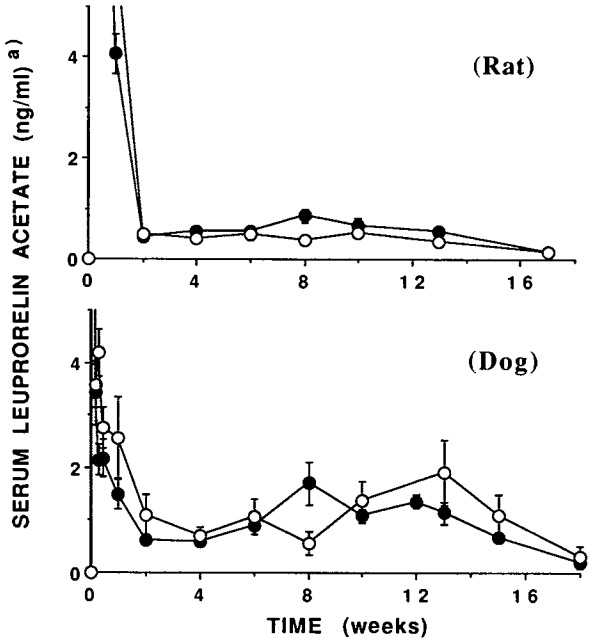


Fig. 3 Serum leuprorelin levels in rats and dogs after s.c. (○) and i.m. (●) injection of the three-month depot. (Dose = 100 (rat) and 25.6 (dog) μg/kg/day, mean±SE, n = 5) a) C_{3h} = rat: 154.2±15.7 (s.c.), 102.4±10.4 (i.m.); dog: 55.7±8.5 (s.c.), 64.2±8.2 (i.m.); C_{1w} = rat: 6.2±0.6 (s.c.) ng/ml.

Although these doses were calculated using the ratio of total body clearance in both animals after injection of the aqueous drug solution to provide the same plateau serum levels (8), the serum levels in rats were lower than those in dogs. The reason for this is obscure, but it is assumed that clearance of the microspheres, such as uptake by macrophages, in rats might be more rapid than that in dogs. These serum plateau levels were slightly lower than those after injection of the one-month depot formulation. The reason for this is also unknown, but it might be ascribed to the rapid clearance of PLA microspheres as compared with PLGA microspheres.

Serum Testosterone, LH and FSH in Rats and Dogs after Injection of the Microspheres

Serum testosterone levels in rats and dogs after s.c. and i.m. injection of the microspheres are shown in Fig. 4. These levels were severely suppressed for 17-18 weeks by a single injection of this depot formulation at a dose of 100 (rat) or 25.6 (dog) μg/kg/day of the drug and amounted to less than one-fifth of those in untreated normal controls (2-4 ng/ml) similarly by both administration routes (9). Serum testosterone was suppressed dose-dependently in rats, and maximal inhibition was attained at a dose of 100 μg/kg/day of the drug as is the case with the one-month depot formulation (Fig. 8). Normal testosterone levels were again observed in both animals 20 to 22 weeks after injection.

Fig. 5 shows serum LH and FSH levels in rats after s.c. and i.m. injection of the microspheres at a dose of 100 μg/kg/day of the drug. A single injection of the microspheres caused transient elevation of serum levels of both gonadotropins, due to the agonistic activity of the drug, followed by drastic suppression for 21 weeks. The suppression profiles following s.c. and i.m. injection were identical.

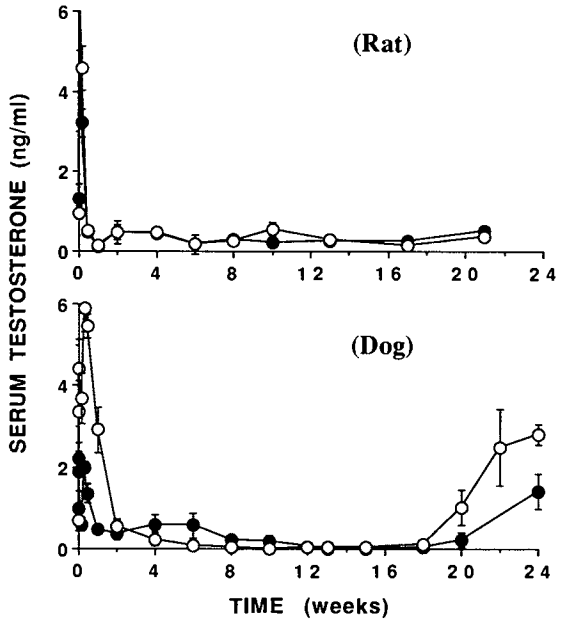


Fig. 4 Serum testosterone levels in rats and dogs after s.c. (○) and i.m. (●) injection of leuprorelin three-month depot. (Dose = 100 (rat) and 25.6 (dog) μg/kg/day, mean±SE, n = 5)

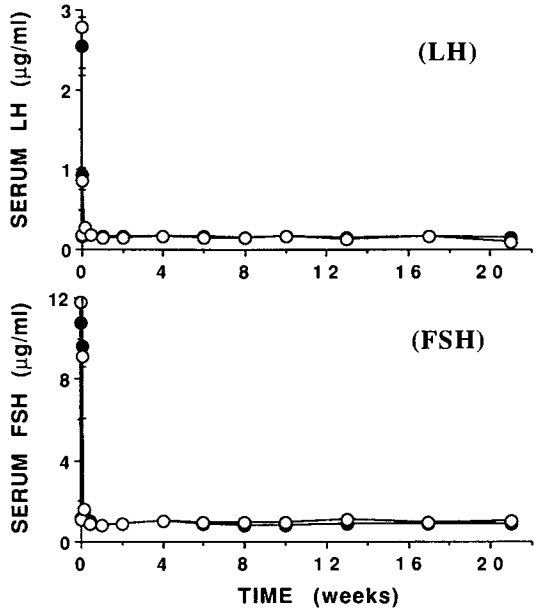


Fig. 5 Serum levels of LH and FSH in rats after s.c. (○) and i.m. (●) injection of leuprorelin three-month depot. (Dose = 100 μg/kg/day, mean±SE, n = 5)

Testosterone, LH and FSH Release in Rats upon a Challenge Test after Injection of the Microspheres

Fig. 6 shows the testosterone release responses following periodic injection of leuprorelin acetate solution in untreated control rats and in rats treated with microspheres at a dose of 100 μg/kg/day of the drug before the challenge.

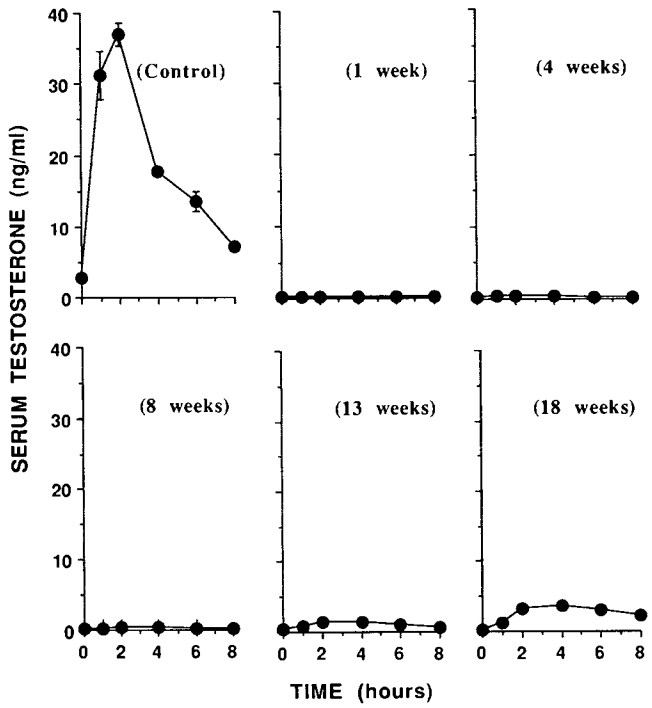


Fig. 6 Serum testosterone in rats following challenge with leuprorelin aqueous solution at different times after i.m. injection of leuprorelin three-month depot. (Dose = 100 μg/kg (solution) and 4.5 mg/rat (microspheres), mean±SE, n = 5)

Testosterone release was inhibited dramaticatlly for 13 weeks.

Fig. 7 shows LH, FSH and testosterone release, increment in the AUC (area under the serum level-time curve), following a challenge with leuprorelin solution in untreated control rats and in rats treated with the microspheres before the challenge. Testosterone, LH and FSH release were dramatically depressed for 15 weeks; all values were significantly lower than those in the untreated control rats ($P<0.001$). This clarified that persistent inhibition of the pituitary-gonadal axis for over 13 weeks is caused by a single injection of the microspheres just as dramatically as in the case of three treatments with the one-month depot formulation (9). Partial recovery of the function of the pituitary-gonadal system was seen 18 and 21 weeks after injection, but the function was still significantly suppressed ($p<0.001$).

Suppression of Genital Organ Growth after Injection of the Microspheres

Fig. 8 shows the suppression of growth of the genital organs after a single injection of the microspheres at different doses. The growth of the testes was significantly inhibited even by the lowest dose (1 μg/kg/day); the highest dose (100 μg/kg/day) elicited the strongest inhibition for 18 weeks. This suppression profile correlated fairly well with that for serum testosterone. Growth of both the seminal vesicles and prostate was depressed dose-dependently after a slight initial increase owing to the agonistic activity of the drug. The lowest dose was insufficient for suppression, but 10 and 30

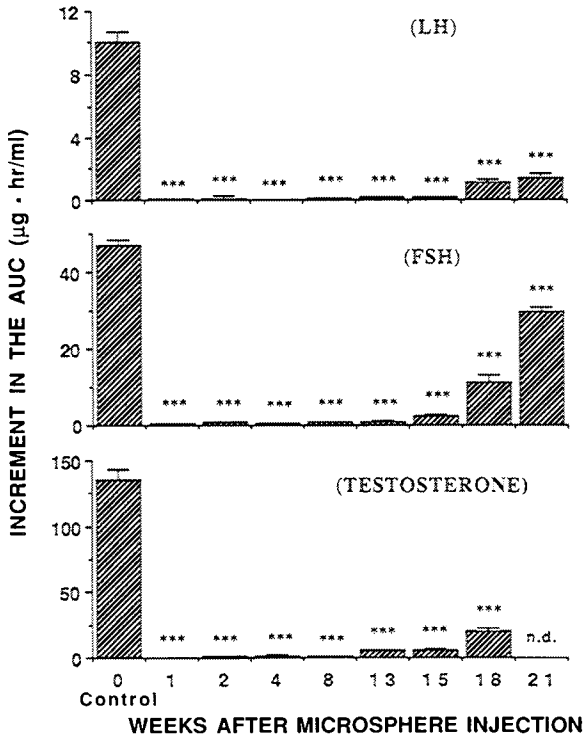


Fig. 7 Increment in the AUC of serum LH, FSH and testosterone for 8 hr in rats following challenge with leuprorelin aqueous solution at different times after i.m. injection of the three-month depot. The doses were the same as those indicated in Fig. 6. (mean±SE, n = 5, ***: $p<0.001$ (Student's t-test), n.d., not determined)

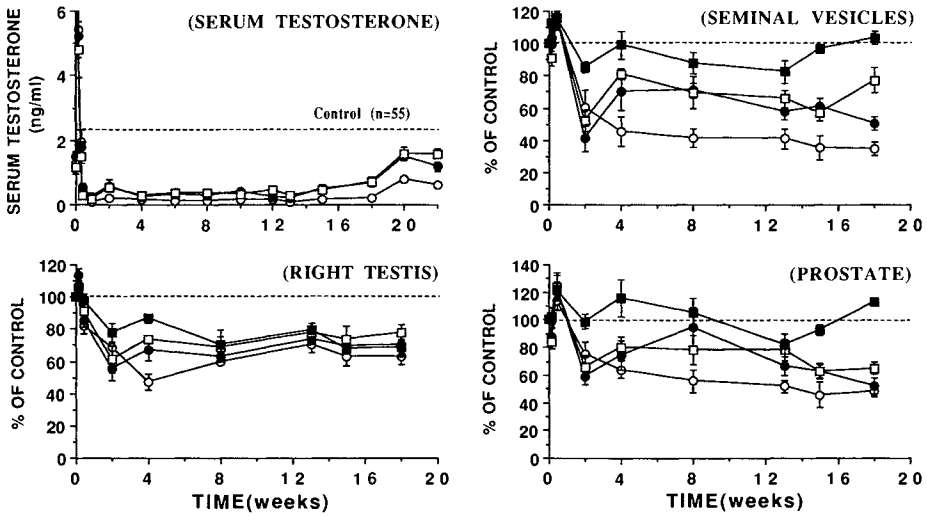


Fig. 8 Serum testosterone and genital organ weight change in rats after injection of leuprorelin three-month depot. (Dose = 100 (○), 30 (●), 10 (□), 1 (■) μg/kg/day, mean±SE, n = 5)

μg/kg/day provided obvious inhibition for over 13 weeks. In all of these reproductive organs, the strongest inhibition was achieved at a dose of 100 μg/kg/day of the drug as with the one-month depot formulation (10,11). The duration of genital organ growth inhibition after injection of the microspheres coincided well with the duration of drug release and that of the suppression of steroidogenesis in the testes.

In summary, the microspheres of leuprorelin acetate prepared with PLA having a m.w. of about 15,500 released the drug almost linearly for 13 weeks (3 months) after s.c. and i.m. injection, and provided a sustained serum drug level and inhibited steroidogenesis dramatically in rats and dogs. A single injection of the microspheres caused drastic suppression of the function of the pituitary-gonadal axis and inhibited the growth of the reproductive organs in rats at a dose of 100 μg/kg/day for over 3 months. It was concluded that persistent pharmacological effects equivalent to those obtained upon chronic treatment using the once-a-month formulation were achieved after a single injection of this three-month depot formulation. Thus, this depot formulation would be more convenient than the one-month depot formulation, and this should assure greater patient compliance which would result in more reliable therapeutic effects in patients suffering from hormone-dependent diseases.

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