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Assessment of Fertility in Male Rats After Extended Chemical Castration with a GnRH Antagonist

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Susan S. D'Souza,[1] Francesca Selmin,[2] Santos B Murty,[3] Wei Qiu,[1] BC Thanoo,[4] and Patrick P DeLuca[5]

[1]University of Kentucky College of Pharmacy, Lexington, KY 40536
[2]Istituto di Chimica Farmaceutica e Tossicologica, Universita' degli Studi di Milano, Milano, Italy
[3]Murty Pharmaceuticals, Lexington, KY
[4]Oakwood Labs, Oakwood, OH
[5]Faculty of Pharmaceutical Sciences, University of Kentucky College of Pharmacy, Lexington, KY 40536

ABSTRACT

The purpose of this study was to assess whether male rats whose testosterone levels were suppressed to castration levels (<0.5 ng/mL) for a 1-year period by the sustained delivery of orntide acetate, a GnRH antagonist, would return to fertility (ie, produce offspring) after serum testosterone returned to control levels. Male rats comprising a treatment group (orntide microspheres, dose = 27 mg/kg/y), a vehicle control group, and a control group of proven male breeders were used. For the treatment and vehicle control groups, serum orntide and testosterone levels were monitored at periodic intervals for 14 months from the initiation of treatment. After serum testosterone levels returned to vehicle control levels and orntide serum levels were no longer discernible for the treated group, each of the animals was housed with 2 drug-naive, female, proven breeders. All the breeder females produced offspring with the exception of 1 female housed with a male rat from the treatment group and the 2 females housed with a single male rat from the vehicle control group. The mean size and weight of the litters from each group were not statistically different. Further, fertility of the offspring from each group was assessed. The male and female offspring studied were all shown to be fertile. The results suggest that lack of fertility due to testosterone suppression in male rats is reversible after cessation of treatment with the GnRH analog, orntide.

KEYWORDS: orntide acetate, PLA microspheres, return to fertility, chemical castration, GnRH antagonist

Corresponding Author: Patrick P. DeLuca, Faculty of Pharmaceutical Sciences, University of Kentucky College of Pharmacy, Lexington, KY 40536.
Tel: (859) 257-1831. Fax: (859) 323-0242.
Email: ppdelu1@uky.edu.

INTRODUCTION

GnRH superagonists like leuprorelin, triptorelin, goserelin, and buserelin currently administered as long-acting dosage forms,[1-4] act by hyperstimulation and downregulation of GnRH receptors on the gonadotroph cells of the pituitary. These peptides are used for treatment of a variety of hormone-dependent diseases like prostate cancer and endometriosis. Unfortunately, the superagonists stimulate luteinizing hormone-releasing hormone (LHRH) production, which in turn causes a surge of testosterone and androgens resulting in a "clinical flare." Eventually, they downregulate receptors, block release of LH and follicle stimulating hormone (FSH), and also prevent synthesis/release of testosterone or estrogen from the gonads.[5-7] The superagonists have been successfully used in patients, and their safety record is well established. In addition, upon cessation of therapy with the superagonists, these patients are able to bear children. Therefore, return to fertility with commercially available GnRH superagonists is not a clinical concern.

Another class of GnRH analogs, the antagonists, are being investigated as potential peptide therapeutics.[8-9] These antagonists nullify the action of native GnRH peptides by binding to the GnRH receptors without eliciting a pharmacological response and have been shown to be devoid of the initial-androgen stimulation characteristics of the superagonists, which appears to be a significant benefit over the superagonists.[10-11]

Efforts to administer peptide therapeutics via the oral route are hindered by their poor bioavailability due to enzymatic degradation and to poor passage through biological membranes because of a low partition coefficient and poor diffusivity. Achieving constant levels of bioactive peptides in vivo would require a controlled rate of administration of the therapeutic agent. Formulation of peptides as microspheres, using biodegradable polymers, has been shown to afford controlled delivery of these agents. Biodegradable microspheres are injectable dos-

Table 1. Fertility Study Design (F_0 Generation)*

Group	Group Description (Age in Months)	Treatment	Orntide Dose (mg/kg)	No. of Males
I	Treated males (17.5)	Orntide microspheres	27	6
II	Untreated males (17.5)	Vehicle control	-	5**
III	Young proven male breeders (5)	Drug naive	-	5

*Study conducted with young female proven breeders (age, 5 months).
**Of the 6 males in the vehicle-control group of the testosterone suppression study, one died at 390 days, presumably of old age.

age forms fabricated from biodegradable and biocompatible polymers such as polylactide (PLA) and poly(lactide-co-glycolide), which have been used as surgical sutures for more than 30 years and are approved for human use by the Food and Drug Administration. These systems improve patient compliance by eliminating the need for frequent administration of peptide.[12,13] In vivo, peptide release from these biodegradable microspheres occurs as a result of hydration, which leads to polymer erosion or degradation.[14-15] For this reason, controlled-release depot formulations of orntide, a potent GnRH antagonist, are being developed. These formulations sustain drug levels in vivo for varying periods of time.[16]

A previously published study showed that male rats treated with 120-day orntide microspheres were able to produce offspring upon cessation of treatment.[17] The goal of the present study was to assess the return to fertility in male rats, post treatment of a 1-year PLA microsphere dosage form of orntide, in comparison to untreated vehicle control and younger breeders. In addition, effects on fertility of the second generation were determined.

MATERIALS AND METHODS

Materials

Orntide acetate was supplied by California Peptide Research Inc (Napa, CA) and was 99% pure. Polylactide (PLA) polymer (MW = 22 kDa) from Boehringer Ingelheim (Ingelheim, Germany) was used to formulate the microspheres. Orntide microspheres were prepared as previously described by solvent extraction.[14]

Three-month-old, male, Sprague-Dawley rats, purchased from Harlan (Indianapolis, IN), weighing approximately 300 g were used to study the testosterone suppression due to orntide administration in microspheres (treated group). The male rats were acclimatized to the housing facility for 1 week, prior to administration of microspheres and vehicle control. Five-month-old, proven male and female breeders (Sprague Dawley) were purchased from the same supplier and used to assess fertility.

All the procedures were approved by the Department of Laboratory Animal Research at the University of Kentucky, Lexington, KY.

In Vivo Evaluation of Orntide Microspheres

Two groups of male Sprague-Dawley rats (n = 6) were used in this study. Group I received the 1-year orntide microsphere formulation (27 mg orntide/kg/y) prepared with the polymer in vehicle (1% sodium carboxymethylcellulose, 2% mannitol, and 0.01% Tween 80). Animals in Group II were used as a vehicle control and received vehicle alone. The injections were administered subcutaneously using a 20-gauge needle at the base of the neck. Blood samples were collected from the tail vein at predetermined intervals. The serum was separated and frozen until analysis. Following the 1-year treatment period, the orntide microsphere-treated rats were subjected to a washout period until they resumed and maintained similar testosterone levels as the vehicle control.

Serum orntide levels in rats were measured using a radioimmunoassay.[15] Tyrl-orntide was radioiodinated by the lactoperoxidase method and the labeled ligand was purified by high-performance liquid chromatography (HPLC). Orntide was conjugated by the carbodiimide method, and the antibody to orntide was produced in rabbits. The lower detection limit of the assay was 0.008 ng/mL. The intra- and interassay coefficients of variation were 6% and 9%, respectively.

Serum testosterone levels were assayed using Active Testosterone RIA DSL-4000 kits (Diagnostic Systems Inc, Webster, TX). The lower detection limit for this assay was 0.08 ng/mL and the intra- and interassay coefficients of variation were 10% and 9%, respectively.

Fertility Study (F_0 Generation)

Testosterone levels in male rats from Group I returned to normal (similar to Group II) by 390 days. At 438 days, after allowing an additional washout period, the males were housed with 2 proven breeder females for 1 month (Table 1). A proven female breeder is defined as having

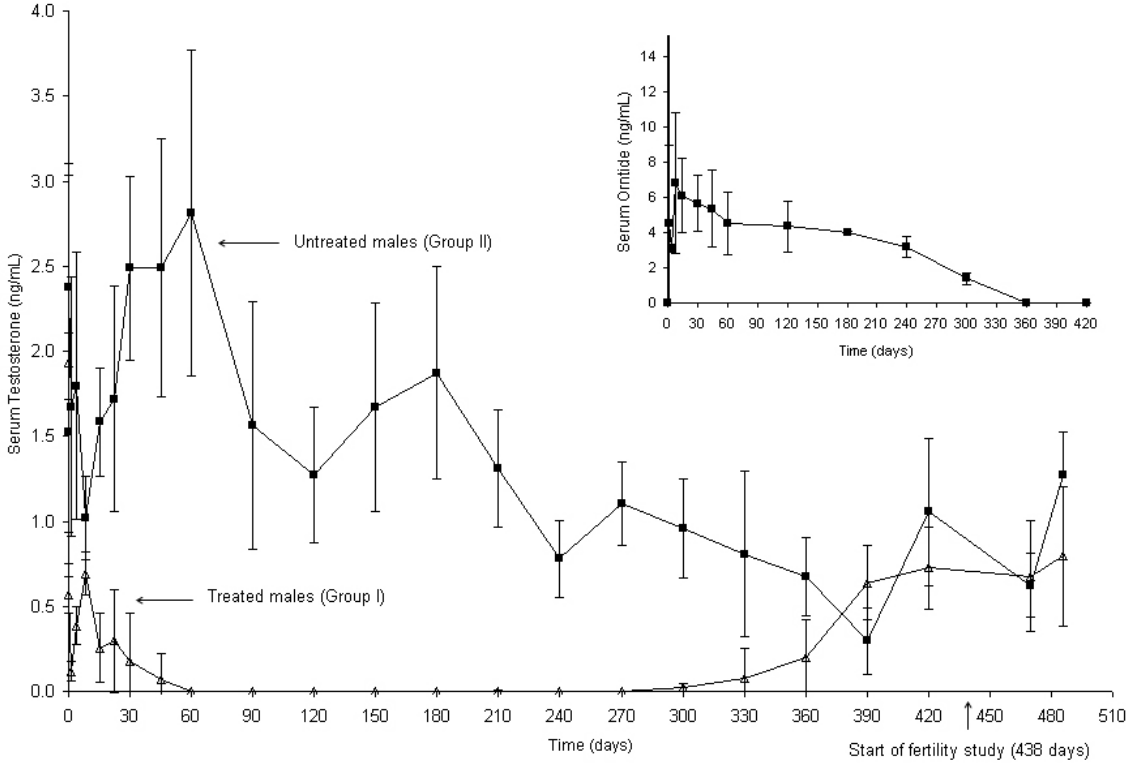

Figure 1. Testosterone levels for treated and vehicle-control groups. The insert shows the serum orntide levels.

given birth to at least 2 offspring. Drug-naive male proven breeders (Group III) were also each housed with 2 female breeders to assess the effect of age on the ability of male rats to sire offspring. Pregnant females were separated from males and housed in separate cages. After birth, the number of offspring (males and females) was recorded and the offspring were observed for any abnormality during weaning (ie, 30 days after birth).

Fertility Study (F_1 Generation)

After weaning, 3 offspring males were randomly selected from each of the 3 F_0 generation Groups (I-III). Each male was housed with 2 females, a female offspring from the F_0 generation and a proven mother that was used as a positive control. These three groups are designated as Groups IV-VI. F_0-generation female offspring were selected to assess fertility within and between groups. Pregnant females were separated from males and housed in separate cages. After birth, the number of offspring (males and females) was recorded and the offspring were observed for any abnormality during weaning (ie, 30 days after birth).

Statistical Evaluation of Data

The data are presented as mean ± standard deviation for all the analyses performed. For values below the assay detection limit, the limit was used for calculations.

RESULTS AND DISCUSSION

Drug Release and Testosterone Suppression

Figure 1 shows the testosterone levels in the treated and vehicle groups. Testosterone levels were suppressed to near 0.5 ng/mL after 6 hours and were below 0.5 ng/mL in 1 day. There was no evidence of an elevation of testosterone, which occurs with the LHRH superagonists. The initial burst release of orntide led to a sharp drop in testosterone levels followed by a slight elevation, which is concurrent with the hydration phase for these microspheres. Testosterone levels remained at approximately 0.5 ng/mL for 8 days and then gradually declined to nondetectable levels by day 60. Full chemical castration was achieved from day 15 through day 360 for this formulation.

The insert in Figure 1 shows the in vivo drug levels obtained postadministration of microspheres in the Group I

Table 2. F_0-Generation Offspring Produced by Male Rats From Groups I to III and Female Proven Breeders

Group (Description)	Male, No.	Number of Offspring			
		Female No. 1		Female No. 2	
		Male	Female	Male	Female
I (Males treated with orntide microspheres)	1	11	3	2	1
	2	5	5	6	6
	3	8	4	5	9
	4	*	*	**	**
	5	7	6	4	8
	6	7	5	7	5
II (Vehicle-control males)	1	**	**	**	**
	2	6	9	8	5
	3	8	4	6	3
	4	4	8	6	5
	5	7	6	8	6
III (Drug-naive, male proven breeders)	1	5	10	3	5
	2	5	10	10	4
	3	8	5	4	6
	4	4	6	7	5
	5	6	6	5	5

*Animals sacrificed shortly after birth due to mastitis in the mother. Hence, sex of the offspring was indeterminable. Litter size was 11.
**No offspring.

animals. An initial burst (C_{max} = 75 ng/mL at 6 hours) of orntide from the microspheres caused a rapid drop in serum testosterone levels to below chemical-castration level (defined as serum testosterone <0.5 ng/mL). The initial burst could be due to the release of peptide associated with the surface of the microspheres. Following this initial burst, serum peptide levels fell sharply, and levels remained at 3 to 4 ng/mL up to day 4. This finding may be attributed to the time required to hydrate the polymer. By day 8, peptide levels rose approximately 7 ng/mL and then remained above 4 ng/mL through 180 days. Orntide levels declined gradually but were detectable until day 360.

Serum analysis of the vehicle control (data not shown) showed no presence of the drug, and this was reflected in the testosterone levels (Figure 1). After 390 days, testosterone levels for the vehicle control and treated groups were similar.

Fertility Study

Figure 1 shows that baseline orntide levels occurred at 360 and 420 days, and while testosterone levels of treated and vehicle-control males were similar at 390 days, an additional washout period was allowed (study started at 438 days). Each treated and vehicle-control male was housed with 2 female proven breeders to assess fertility. Young, male proven breeders were also used and housed with female proven breeders in a manner similar to the other groups.

All the male rats treated with orntide microspheres sired offspring with at least 1 female proven breeder (Table 2). The female proven breeders had an average of 12 offspring, similar to Group III (offspring of male proven breeders). One male from the vehicle-control group failed to sire any offspring with either female. The reason for this could be disinterest or the inability of the female to produce offspring. To assess the fertility of a nonmother (female that did not produce any offspring), it was housed with a young, drug-naive, male proven breeder. Also included in the same cage was a proven mother to assess the young male's ability to sire offspring. The other 8 females housed with Group II males produced from 9 to 15 pups or an average of 12. There was no significant difference in the number of offspring produced by the treated rats in comparison with the vehicle-control group and the drug-naive, male proven breeders. All the offspring appeared normal, with no physical deformities.

Atkinson et al, in a 2-generation reproductive and fertility study, reported a fertility index for proven breeder

Table 3. F_1-Generation Offspring Produced by the F_0-Generation Offspring From Groups I to III

Group (Description)	F_0-Generation Male Offspring, No.	Number of Offspring			
		F_0-Generation Female Offspring		Female Proven Breeder (Control Group)	
		Male	Female	Male	Female
IV	1	8	9	6	5
(Offspring of males treated with orntide microspheres, Group I)	2	3	8	*	*
	3	4	1	*	*
V	1	6	9	4	6
(Offspring of vehicle-control males, Group II)	2	3	5	4	5
	3	0	1	5	3
VI	1	4	6	7	5
(Offspring of male proven breeders, Group III)	2	8	6	5	7
	3	4	9	10	4

*No offspring.

females housed with males and a survival index for the offspring at 30 days (see Equations 1-3 below).[18]

$$\text{Fertility Index (Female)} = [\text{No. of Females Delivering} / \text{No. of Females Cohabited}] \times 100 \quad (1)$$

$$\text{Fertility Index (Male)} = [\text{No. of Males Siring Offspring} / \text{Total No. of Males in the Group}] \times 100 \quad (2)$$

$$\text{Survival Index} = [\text{No. of Live Offspring at Day 30} / \text{No. of Live Offspring at Day 0}] \times 100 \quad (3)$$

The fertility indices for males used in the F_0-generation study were 100%, 80%, and 100%, respectively, for Groups I to III. For the females housed with Groups I to III, values of 91.6%, 80%, and 100% were obtained. As expected, the values were lower for Group II (vehicle-control group), in which 2 females did not produce any offspring when housed with a male.

The survival indices for the offspring produced in the F_0 generation were 93.7%, 87.0%, and 99.0% for Groups I to III, respectively.

Fertility of nonmothers was assessed by housing with male proven breeders. The average number of offspring produced in this study was 12 with a range of 10 to 15. Fertility and survival indices for the nonmothers used in this study were both 100%. All of the offspring showed no signs of abnormality at birth.

A second study was initiated to assess the reproductive ability of the F_0 generation to verify if orntide could affect the reproductive ability of the offspring. Similar studies have been performed on rats exposed to agents like mercuric chloride[18] and butylated hydroxytoluene (BHT),[19] and the differences between the 2 generations have been reported.

F_0-generation offspring males from Groups I to III were able to sire pups with female offspring from the same generation (Table 3). Therefore, it may be concluded that treatment of one generation of male rats with orntide does not affect the reproductive ability of their male and female offspring. Fertility indices were 100% for the male and female offspring used in this study. The results of the fertility and survival indices for both generations are shown in Table 4. Also, there is no significant difference in the number of offspring produced between the Groups I to VI.

For offspring from the F_0 and F_1 generations, weight was recorded at the time of weaning (ie, 30 days). Weight gain is frequently used as an indicator of general health and development.[18] At the time of weaning, there was no significant difference in the weights of the litters between the Groups I to VI, indicating that orntide treatment did not affect the developmental growth of the offspring.

CONCLUSION

A depot formulation of orntide, a GnRH antagonist, appeared to chemically castrate male rats for a 1-year period (testosterone < 0.5 ng/mL). Adult male rats that had been chemically castrated for a 12-month period were able to return to fertility and the number and weight of offspring produced were not statistically different from the vehicle control and young proven breeders. The re

Table 4. Fertility and Survival Indices

Group	Fertility Index (%)		Survival Index for Offspring (%)
	Males	Females	
I (Males treated with orntide microspheres)	100	91.6	93.7
II (Vehicle-control males)	80.0	80.0	87.0
III (Drug-naive male proven breeders)	100	100	99.0
IV (Offspring of males treated with orntide microspheres, Group I)	100	66.6*	100
V (Offspring of vehicle-control males, Group II)	100	100*	93.6
VI (Offspring of male proven breeders, Group III)	100	100*	100

*Data presented are for offspring females (F_0 generation).

productive ability of chemically castrated rats was preserved despite their age. This finding supports the use of the GnRH antagonist, orntide, in patients who wish to regain their capacity for reproduction after hormonal treatment.

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