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Recovery of Pituitary-Gonadal Function in Male Rats after Long-Term Suppression Induced by a Single Injection of Microcapsules of LH-RH Antagonist Cetrorelix (SB-75)

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ABSTRACT: The clinical utility of luteinizing hormone-releasing hormone (LH-RH) analogs can be greatly enhanced by a sustained delivery system, which could maintain elevated peptide levels in the blood for prolonged periods of time, up to several weeks. Recently, we developed long-acting microcapsules and microgranules of the LH-RH antagonist SB-75. In this study, we examined the suppressive effects of a single injection of microcapsules of antagonist SB-75 on gonadotropin and testosterone secretion, as well as on fertility, in male rats and the reversibility of those effects. Serum SB-75 levels were measured by RIA. A dose of 20 mg of microcapsules/rat containing 3.58 mg of antagonist in poly(D,L-lactide-co-glycolide), administered intramuscularly produced SB-75 levels higher than 20 ng/ml for approximately 24 days, and a significant elevation was maintained until day 90. Serum testosterone was decreased to castration values for 164 days and LH levels were suppressed below the detection limit of the RIA for a period of 102 days. Serum FSH was suppressed by more than 90%, as compared to control animals, for a period of 58 days and remained significantly decreased until day 164 after the injection. This treatment also caused a significant decrease in the weights of the testes, seminal vesicles, and ventral prostate 30 days after peptide administration. The histology of the testes from the treated rats showed that spermatogenesis was totally depressed. No mature elongated or round spermatids were found in the seminiferous tubules, spermatocytes being the most advanced germ cell form in 99.5% of the testicular tubules. Ten months after injection, a complete recovery in organ weights, hormonal levels, and fertility was observed. Histological studies revealed a complete recovery of spermatogenesis, with 100% of seminiferous tubules containing mature elongated spermatids. All treated rats proved to be able to impregnate normal female rats. The offspring were normal, with no evidence of genetic abnormalities. The overall results demonstrate the efficacy of SB-75 microcapsules in suppressing the pituitary–gonadal axis for a prolonged period of time and show that the long-term suppression of gonadal function induced by chronic treatment with antagonist SB-75 is completely reversible.

Key words: Reversible gonadal inhibition, cancer, contraception.
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During the past 20 years, more than 3,000 analogs of luteinizing hormone-releasing hormone (LH-RH) have been synthesized and evaluated for possible therapeutic use (Schally et al, 1984, 1989; Karten and Rivier, 1986). There is much clinical evidence that, in addition to various gynecological applications, LH-RH agonists can be used for treatment of advanced prostate cancer (Tolis et al, 1982; Nicholson et al, 1984; Redding et al, 1984; The Leuprolide Study Group, 1984; Parmar et al, 1985; Ahmann et al, 1987; Crawford et al, 1989; Sharifi and Soloway, 1990). Attempts have also been made to inhibit fertility in human beings with LH-RH analogs (Schally et al, 1989; Behre et al, 1992). While repeated administration of LH-RH agonists is necessary for inhibition of LH and sex hormones, similar effects can be induced with a single injection of a potent LH-RH antagonist (Schally et al, 1984; Karten and Rivier, 1986; Schally et al, 1989; Pinski et al, 1992a,b). New LH-RH antagonists, free of edematogenic and anaphylactoid reactions, were synthesized in our laboratory and tested *in vitro* and *in vivo* (Bajusz et al, 1988a,b). Among these analogs [Ac-D-Nal(2)^1, D-Phe(4Cl)^2, D-Pal(3)^3, D-Cit^6, D-Ala^{10}]-LH-RH (Cetrorelix, SB-75) proved to be the most potent, as judged by the inhibition of LH release *in vitro*, blockade of ovulation in cycling rats, and suppression of LH levels in ovariectomized rats in doses as small as 0.625 μg (Bajusz et al, 1988a,b).

The clinical utility of these compounds can be greatly enhanced by a sustained delivery system, which elevates peptide levels in the blood for prolonged periods of time. Consequently, long-acting delivery systems in microcap-

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sules of poly(DL-lactide-co-glycolide) (PLG) designed to release a controlled dose of the peptide over a 30-day period were developed for several agonists and the antagonist SB-75 (Parmar et al, 1985; Redding and Schally, 1990). Clinical efficacy was established for microcapsule formulations of LH-RH agonists in the treatment of advanced prostate cancer (Parmar et al, 1985; Ahmann et al, 1987; Sharifi and Soloway, 1990). However, long-term evaluation of the biological effects of treatment with LH-RH antagonist SB-75 and the demonstration that inhibition of the pituitary–gonadal axis is reversible are necessary before other clinical trials can be started. This paper describes prolonged inhibition of the pituitary–gonadal axis in male rats after a single injection of microcapsules of the antagonist SB-75, as well as the recovery of testicular function and fertility.

Materials and Methods

Animals

Young adult male Sprague-Dawley rats (220–240 g) (Charles River) were used in this study (10 in each group). Animals were allowed standard rat diet and tap water *ad libitum* and were maintained under controlled conditions: 12 hours light/12 hours dark schedule at 24 ± 2°C.

Peptides and Microcapsules

[*N*-Ac-3-(2-naphthyl)-D-alanine^1, 4-chloro-D-phenylalanine^2, 3-(3-pyridyl)-D-alanine^3, D-citrulline^6, D-alanine^{10}]-LH-RH (Cetrorelix, SB-75) was synthesized by solid-phase methods in our laboratory as well as by Asta-Medica (Frankfurt/Main, Germany) and carefully repurified by HPLC. Microcapsules of SB-75 pamoate (lot RCSES-91-07M) were prepared by Dr. P. Orsolini (Debio Recherche, Martigny, Switzerland) and consisted of SB-75 pamoate (17.9%) distributed within a polymer matrix of poly(DL-lactide-co-glycolide). For injection, the microcapsules were suspended in 0.7 ml of injection vehicle consisting of 2% CM-cellulose and 1% Tween 80 in water. The suspension was mixed thoroughly on a vortex mixer and injected intramuscularly (i.m.) through an 18-gauge needle.

Hormone Determination

Serum LH and FSH were determined by specific radioimmunoassays with material supplied by the National Hormone and Pituitary Program (Niswender et al, 1968). Testosterone levels were measured with a kit provided by Diagnostic Products (Los Angeles). SB-75 was determined by RIA using a highly specific antibody developed in our laboratory (Csernus et al, 1990a). All hormone estimations were performed as a batch in the same assay. Intra-assay variation was <10%, and inter-assay was <15%. The results are expressed as mean ± SEM. Statistical significance was determined by one-way analysis of variance (ANOVA). In all analyses, differences were considered significant if $P < 0.05$.

Fertility Test

Nine months after injection with SB-75 microcapsules, treated male rats and female rats (290–320 g) on the day of proestrus were assigned to mating chambers. Each mating chamber contained one treated male rat and three female rats. Reproductive cyclicity of all female rats was examined with daily vaginal smears, and the day of conception (day 1) was determined by the presence of sperm in the vaginal lavage. Fertility was assessed by counting the number of rats in each chamber giving birth at term.

Histological Procedures

Testicles were fixed in buffered neutral formalin and embedded in Paraplast (Oxford Labware, St. Louis, MO). Six-micron sections were cut and stained with hematoxylin and eosin. Testicular histological changes were classified according to the percentage of tubules containing the most advanced germ cell type (i.e., elongated and round spermatids, spermatocytes, spermatogonia, or Sertoli cells only) (Bokser et al, 1991). All tubules in one cross section of the testicle of each animal were analyzed and the results statistically evaluated.

Experimental Procedures

Microcapsules of SB-75 (lot RCSES 91-07M, core load 17.9%) were injected i.m. at a dose of 20 mg/rat into young adult male rats. This dose was equivalent to 3.58 mg SB-75/rat. Control animals received only vehicle injections. Ten rats were used in each group. Blood samples of 250–500 μl were taken from the tail vein into capillary tubes every day during the first week and twice a week thereafter. Serum was separated by centrifugation, and the supernatant was diluted 1:1 with phosphate buffer containing 0.5% sodium azide. One month after the injection, and at the end of the experiment (10 months after the injection), five rats from each group were sacrificed by decapitation. Testicles, ventral prostate, and seminal vesicles were then removed and weighed.

Results

Serum SB-75 levels in rats injected with microcapsules of SB-75 are shown in Figure 1a,b. Three hours following the injection of 20-mg microcapsules, SB-75 levels increased to 47.1 ± 6.7 ng/ml and remained at that level (42.1 ± 2.5 ng/ml) until day 10 (Fig. 1a). There was only a slight decrease in SB-75 levels (29.8 ± 1.7 ng/ml) 14 days after the injection. This very high serum concentration of SB-75 was maintained until day 24 (Fig. 1a). Thereafter, the peptide level declined slowly to 9.0 ± 1.6 ng/ml on day 49 and continued to fall to 4.4 ± 0.4 ng/ml on day 89 (Fig. 1b). Serum blank of control rats was in the range 0.3 ± 0.02 to 0.7 ± 0.1 ng/ml throughout the experiment.

After administration of microcapsules of SB-75, serum testosterone levels fell below the detection limit of the RIA (0.04 ng/ml) during the first 24 hours and remained below the castration limit (0.5 ng/ml) until day 164 (Fig.

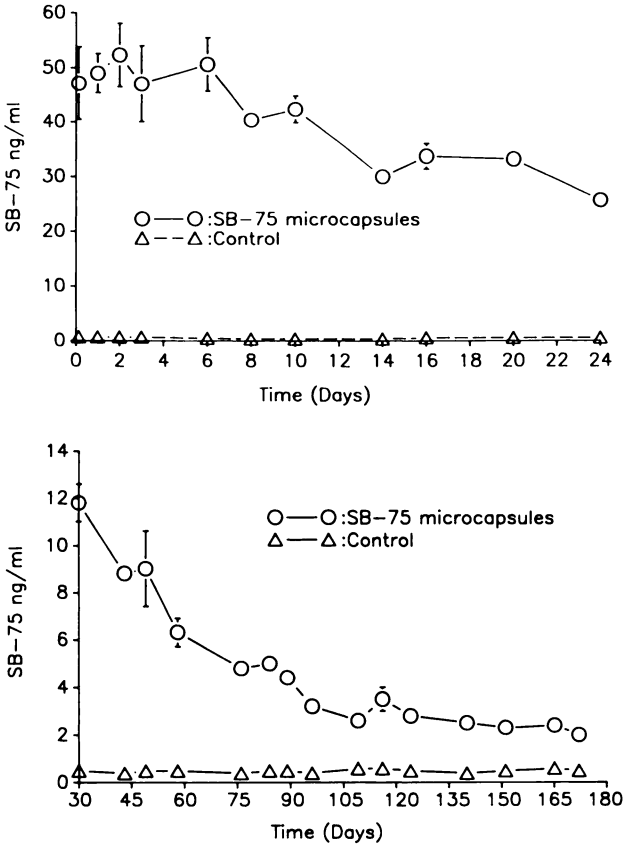

FIG. 1. Serum SB-75 levels in rats, 3 hours (day 0) to 24 days **(a)** and 30–170 days **(b)** after i.m. injection of 20-mg/rat microcapsules of SB-75. Vertical bars show SEM.

2). Testosterone levels in control animals were significantly higher ($P < 0.005$) than in treated animals for 230 days. Serum LH levels were suppressed below the detection limit of the RIA (0.08 ng/ml) for a period of 102 days and returned to control levels by day 151 (Fig. 3). Serum FSH was suppressed by more than 90%, compared to control animals, for a period of 58 days and remained

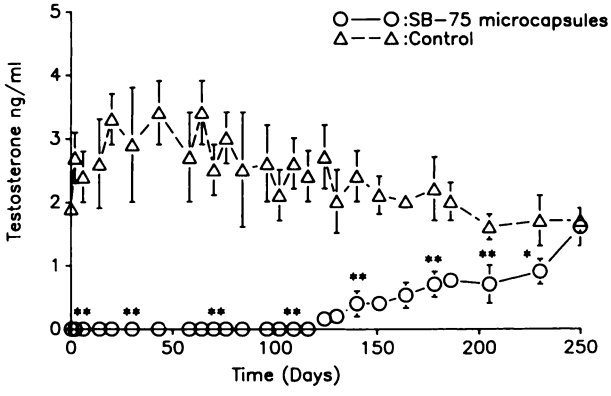

FIG. 2. Serum testosterone levels in rats after i.m. injection of 20-mg microcapsules of SB-75. First measurement (day 0) was performed 3 hours after injection. Vertical bars show SEM. $*P < 0.005$; $**P < 0.001$ vs. control.

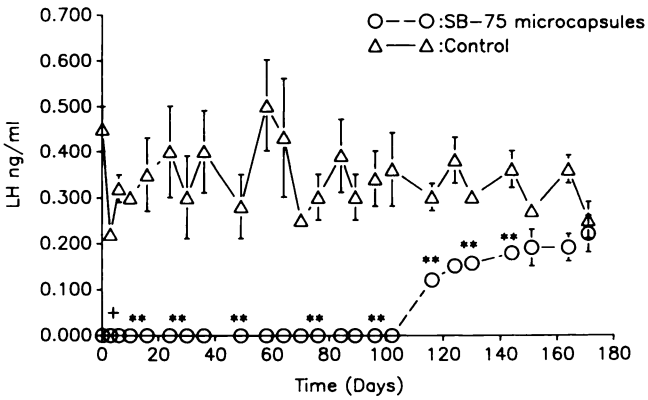

FIG. 3. Serum LH levels in rats after i.m. injection of 20-mg/rat microcapsules of SB-75. First measurement (day 0) was performed 3 hours after the injection. Vertical bars show SEM. $**P < 0.001$ vs. control.

significantly reduced until day 164 after the injection (Fig. 4).

The effects of SB-75 microcapsules on body and sex organ weights are shown in Table 1. The body weights of the experimental group did not differ from their controls. Thirty days after injection, a significant ($P < 0.001$) decrease in weights of testes, ventral prostate, and seminal vesicles was observed in treated animals. Ten months after peptide administration, the treated animals showed a complete recovery in the weights of the sex organs. The histology of the testes from rats treated with SB-75 for 30 days showed a complete suppression of spermatogenesis. The diameters of seminiferous tubules decreased, and the interstitium became slightly edematous. Spermatocytes were the most advanced cell form in all the tubules after treatment with SB-75. The Leydig cells showed no alterations. The testicles of control animals contained normal tubules. Histology of testicles from animals sacrificed 10 months after treatment with SB-75 showed a complete recovery. All the tubules contained mature elongated spermatids. In the control animals, sacrificed together with

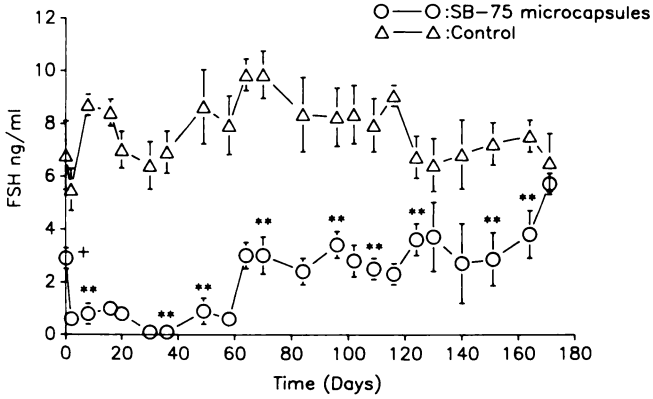

FIG. 4. Serum FSH levels in rats after i.m. injection of 20-mg/rat microcapsules of SB-75. First measurements (day 0) was performed 3 hours after the injection. Vertical bars show SEM. $**P < 0.001$ vs. control.

the latter group, normal histologic structure was found in the testes. The data are shown in Table 2. When the fertility of the rats was checked 9 months after peptide administration, all of the treated rats proved to be fertile and able to impregnate normal female rats. At least one female rat from each mating chamber was found to be pregnant. The offspring were normal, with no apparent evidence of genetic abnormalities.

Discussion

During the past 8 years, considerable effort has been put into the design of microcapsules and implant formulations for sustained release of peptides. Various sustained delivery systems of LH-RH agonists (Decapeptyl, Goserelin, Leuprolide, and Buserelin) have been prepared and applied clinically for the treatment of prostate cancer, breast cancer, endometriosis, leiomyomas, and precocious puberty (Schally et al, 1984, 1989). In the case of LH-RH antagonists, such sustained delivery systems may be even more important than for agonists. The advantage of the antagonists is due to the fact that they inhibit LH, FSH, and sex steroids from the start of administration (Redding and Schally, 1983; Pinski et al, 1992a,b). The use of LH-RH antagonists for the treatment of prostate cancers would prevent the transient stimulation of the release of gonadotropins and sex steroids, which occurs initially in response to LH-RH agonists, thus avoiding the temporary clinical "flare-up" (Schally et al, 1984, 1989; Pinski et al, 1992a,b). Our previous work shows that the antagonists are much more efficacious in inhibiting gonadal function in male mice, when administered using continuous delivery systems than by daily injections (Redding and Schally, 1990). Furthermore, multiple daily injections are inconvenient for chronic treatment and may decrease patients' compliance. We also demonstrated that suppression of the pituitary–gonadal axis in rats induced by administration of SB-75 in microcapsules lasted for a longer period of time than that caused by an equivalent dose of nonencapsulated SB-75 (Pinski et al, 1992a). The mechanism of peptide release from sustained delivery systems (microcapsules or microgranules) after intramuscular injection was studied by histological and immunohistochemical approaches (Csernus et al, 1990b). It was determined that diffusion of the peptides from the aqueous channels in PLG was negligible and that peptide release from the PLG microcapsules was controlled mostly by the speed of biodegradation of the polymer matrix (Csernus et al, 1990b). In order to study the pharmacokinetics of SB-75 release from microcapsules, a specific RIA was developed in our laboratory (Csernus et al, 1990a).

Our results demonstrated a nearly constant and sustained release pattern of SB-75 from this formulation of microcapsules. There was no initial rise of SB-75 levels, as seen after administration of previous batches of microcapsules (Bokser et al, 1990). Although edematogenic and anaphylactoid activity of modern LH-RH antagonists is much lower than that of a previous class of D-Arg^6 antagonists, undesirable side effects related to the "burst effect" could possibly occur during the initial phase of treatment. The absence of an initial rise of SB-75 levels not only prevents such side effects but also helps ensure high peptide levels during the therapeutically important continuous phase.

So far, LH-RH agonists, administered in combination with androgens, failed to achieve azoospermia in a high proportion of men participating in contraceptive trials (Bergquist et al, 1979; Nieschlag et al, 1989; Weinbauer et al, 1990; Behre et al, 1992). It has been reported that LH-RH agonists have a blunting effect on suppression of spermatogenesis induced by 19-nortestosterone (Behre et al, 1992). This phenomenon might be attributed to a differential regulation of pituitary LH and FSH secretion during long-term treatment with LH-RH agonists (Behre

Table 1. *Body, testicular, ventral prostate, and seminal vesicles weights in controls and in rats injected with microcapsules of SB-75 at a dose of 20 mg/rat, evaluated 1 and 10 months after peptide administration**

Body and organ	Time (months)	Control (g)	SB-75 treated (g)
Body Weight	1	416.0 ± 10.7	406.2 ± 11.6
	10	850.3 ± 36.2	904.0 ± 24.3
Testicular weight	1	3.5 ± 0.2	0.6 ± 0.03†
	10	3.8 ± 0.2	3.7 ± 0.2
Ventral prostate weight	1	0.8 ± 0.07	0.05 ± 0.01†
	10	1.3 ± 0.1	1.3 ± 0.2
Seminal vesicles weight	1	1.1 ± 0.05	0.09 ± 0.01†
	10	1.5 ± 0.2	1.2 ± 0.2

* Ten rats were used in each group.
† $P < 0.001$ vs. control.

Table 2. *Effect of treatment with SB-75 on rat spermatogenesis analyzed by histology**

	Control	SB-75	Control	SB-75
	After 30 days		After 10 months	
Elongated spermatids	100		97	100
Round spermatids				
Spermatocytes		99.5	2.5	
Spermatogonia		0.5	0.3	
Sertoli cells only			0.2	

*The numbers show percentage of seminiferous tubules according to the most advanced germ cell type they contain. Ten rats were used in each group.

et al, 1992). Whereas LH and testosterone release were effectively suppressed by LH-RH agonists in volunteers receiving the agonists in combination with androgens or in patients with prostate cancer treated only with the agonists, a selective increase in FSH to almost normal levels could be observed after a short initial suppression (Santen et al, 1984; Kwekkeboom et al, 1990; Behre et al, 1992). Our findings demonstrate that a single administration of SB-75 suppressed FSH release by more than 90% for 58 days as compared to controls. Spermatogenesis was completely inhibited when evaluated histologically 30 days after injection of microcapsules. The suppression of ventral prostate, seminal vesicles, and serum testosterone was similar at 30 days to that obtained by castration (data not shown). Recently, it has been shown in monkeys and men that the administration of LH-RH antagonists together with testosterone produced a complete inhibition of spermatogenesis in almost all treated subjects (Weinbauer et al, 1989; Pavlou et al, 1990; Tom et al, 1991). Thus, it appears that for development of a new method of contraception in men, long-term administration of LH-RH antagonists might be more suitable than the agonists.

Our study indicated that FSH release was almost completely inhibited for the first 2 months after injection of microcapsules and then started to increase, whereas LH secretion was completely suppressed for more than 3 months despite only slightly elevated SB-75 serum levels 2 months after injection. Several studies in humans and rats (Grady et al, 1985; Kartun and Schwartz, 1987; Hall et al, 1988) have shown differential suppression of LH and FSH after short-term LH-RH antagonist treatment. These observations suggest different thresholds for LH-RH receptor blockade for FSH release compared to that of LH. However, the detailed mechanism responsible for these phenomena still remains to be elucidated.

Our results indicate that a total suppression of gonadal function for more than 5 months induced by a single injection of SB-75 in the form of microcapsules is completely reversible. Ten months after peptide administration, no differences were found in organ weights and hormonal levels between treated and control animals. Our mating and histological studies proved that fertility returned parallel to the hormonal recovery from SB-75 treatment. These results are in accordance with our previous findings, which demonstrated a complete recovery of the sex organs in male rats treated for 60 days with SB-75, delivered by minipumps (Bokser et al, 1991).

Antagonist SB-75, in microcapsules, inhibited the growth of Dunning R-3327H prostate cancers in rats (Korkut et al, 1991; Yano et al, 1992) and human prostate carcinoma PC-82 in nude mice (Redding et al, 1992). Thus, antagonist SB-75 could be potentially of clinical value in the treatment of prostate cancer. Administration of LH-RH antagonists based on the use of sustained delivery systems of the type described in our study may allow a convenient and efficacious chronic treatment of prostate cancer and other conditions where inhibition of the pituitary–gonadal axis is desirable. In addition, our results support the view that the approach based on antagonists of LH-RH might be feasible for the development of a method of male contraception, although the exact clinical regimens still need to be established.

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