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Report

Testosterone in a Cyclodextrin-Containing Formulation: Behavioral and Physiological Effects of Episode-like Pulses in Rats

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Testosterone, administered in the form of an inclusion complex with 2-hydroxypropyl-β-cyclodextrin by subcutaneous injection, enters the circulation in a manner markedly similar to the natural episodic release by the testes. The effects of a regimen of once-a-day administration of complexed testosterone to adult (castrated or intact) rats and to senescent (intact) rats were investigated. Although this procedure left the castrated animals with concentrations of circulatory hormone far below physiological levels for much of the day, a significant improvement in androgen-sensitive behavior and physiology was obtained. Furthermore, the testosterone effects were more pronounced when high doses were used periodically rather than when the same total amount of testosterone was equally divided among doses. The same supplementation to intact rats intensified androgen-sensitive behavior and physiology over normal levels. In senescent rats uniform pulses of the testosterone complex also improved behavior and physiology. Specifically, spermatogenesis was stimulated and, notably, the treatment increased muscle weight without substantial enlargement of the prostate. Since the testosterone–cyclodextrin complex also can be effectively administered as a sublingual tablet, the data suggest that similar regimens may be recommended for elderly men suffering from decreases in muscle mass.

KEY WORDS: testosterone, buccal administration; testosterone, administration by bolus; cyclodextrin-testosterone; hydroxypropyl-β-cyclodextrin; androgen-substitutional therapy.

INTRODUCTION

Hormones not only convey crucial messages between tissues but also modulate transmission of messages and sensitivity of responses by target tissues. These modulations arise from the effects hormones exert on the synthesis of their circulating carrier proteins and on the number of functional hormonal receptors in target tissues. The pulsatile release of hormones that has been demonstrated for several glands may be a way to encode these modulations and messages into a system in which a single compound is used for all the signaling (1–3). The androgenic system illustrates these features well. The pituitary gland releases luteinizing hormone intermittently (3). The blood level of testosterone, a hormone which is downstream in the cascade from luteinizing hormone, also varies over time; there is a basal level above which the concentration rises a few times a day in episodes lasting approximately an hour (2,4). These episodes may be triggered by conditioning (5). Testosterone maintains normal activity in various aspects of physiology and behavior but also may induce hyperplasia and support neoplastic growth (6,7). Thus, it is of both theoretical and therapeutic interest to find out which of the androgenic effects require a steady level of circulatory hormone and which effects are cued by episodic increases. The effects of steady levels of testosterone can be evaluated since depot preparations with constant testosterone release have been available for some time (8). The effects of episodic release are difficult to address (9,10) since previous pharmaceutical forms of testosterone generate depots in tissues from which hormone is released gradually into the circulation (11). Only recently has a pharmaceutical form of testosterone been developed which allows convenient administration of physiologically meaningful amounts of hormone in a manner which imitates the natural rapid rise and fall of hormonal levels occurring in episodes (12). This pharmaceutical form is based on an inclusion complex of testosterone with 2-hydroxypropyl-β-cyclodextrin. In this complex a molecule of hormone is included in the cavity of the host molecule; the formation or dissociation of such a complex in solution is very rapid. Consequently, the hormone can quickly transfer from the circulating solution into a tissue, while the carrier 2-hydroxypropyl-β-cyclodextrin stays in the solution. Aqueous solutions of the inclusion complex are stable and do not form precipitates upon dilution. Importantly also,

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2-hydroxypropyl-β-cyclodextrin lacks toxicity or irritancy toward tissues; used in another context a dose of 0.5 g/kg/24 hr intravenously was tolerated in a human male (13). In the present work this pharmaceutical form of testosterone was evaluated on rats which had subnormal levels of testosterone, due either to castration (14,15) or to senescence (16–20).

MATERIALS AND METHODS

Animals

Long–Evans rats were individually housed in a facility with constant temperature (20–22°C), humidity (50%), and 12-hr light/12-hr dark cycle; food and water were freely available. The males were sexually experienced. Assignment of animals to groups was random. In the experiments with young adult male rats (150–200 days old), all animals were anesthetized with ether and either castrated ($N = 64$) or sham operated ($N = 12$). In the experiment with senescent rats, 24-month-old gondally intact males ($N = 14$) of the same strain were used. Adult female rats used were ovariectomized under pentobarbital sodium anesthesia and allowed a week's recovery time. Females were induced to estrus by subcutaneous injection of estradiol benzoate (200 μg) followed 48 hr later by injection of progesterone (800 μg); animals were then used within several hours. For details see Refs. 9, 10, and 21.

Testosterone Preparation and Administration

Testosterone (2.5 g) and 2-hydroxypropyl-β-cyclodextrin (25 g; degree of substitution, 6.2) were stirred in distilled water (100 ml) at room temperature for 3 days. The suspension was then filtered through a Millipore filter (0.45 μm). The clear filtrate was diluted to 200 ml with distilled water and freeze-dried. The resulting white nonhygroscopic powder (26.3 g) contained 7.7 ± 0.04% (w/w) ($N = 3$) of testosterone as determined by spectrophotometry ($\epsilon = 16{,}600$ at 250 nm). This powder was dissolved in isotonic saline solution and injected subcutaneously. Both the powder and the solution were stored at room temperature.

Behavior Tests

In aggression tests each experimental male was paired with an intact male of the same age and strain selected at random from the general animal colony ($N = 30$). Interactions of the pair were observed and aggressive behavior was recorded during a 20-min session (22).

To test sexual performance an estrous female was introduced into a cage in which the male had been placed 15 min earlier. Latencies of the first intromission (seconds) and first ejaculation (minutes) were recorded. The session lasted for 30 min.

In tests of sexual motivation, an inaccessible estrous and nonestrous female were separately contained in small mesh-wire cages placed at each end of a large cage (23). Then the male was placed in the large cage for 20 min. The time spent in that half of the large cage which contained the estrous female was recorded (seconds). Further, urinary markings on fields (altogether 600) in the vicinity of the cages of each female were counted.

Physiological Examination

After the behavioral tests, each male was sacrificed with an overdose of pentobarbital sodium. Androgen-sensitive structures of interest (Tables I and II) were excised using ligation to prevent less fluid where applicable, and wet weights were obtained. Note that in Table II weights relative to body weights were given to enable easier comparison with senescent rats of other strains.

Testosterone in Serum

Sixteen of the males were castrated and tested 2 days later for the half-life of the testosterone in serum. Blood samples from rats were obtained by cardiac puncture and testosterone in serum measured by radioimmunoassay using rabbit antiserum supplied by the Department of Pharmacology, Univeristy of Heidelberg, and [^{3}H]testosterone from Amersham-Buchler, Braunschweig. The intrassay coefficient of variation was 4.6%.

Data Analyses

All groups contained six or more animals. Results from the experiment on behavioral/physiological features of the young adult rats were analyzed with a series of 2 × 4 analyses of variance with the schedules (uniform or rhythmic) and testosterone dosage as main factors. In post hoc group comparisons Tukey's HSD ($P < 0.05$) tests were used. Findings from experiments on senescent rats were examined with t tests ($P < 0.05$). Within-group changes in behavior before and after supplementation with testosterone were analyzed, as well as differences between treated and untreated groups. One senescent animal died during the experiment and his data were not included.

RESULTS

The concentration of testosterone in the circulation of intact rats varies with time, rising above baseline levels in irregularly spaced episodes which occur a few times each day. A typical episode (2) is shown in the left panel in Fig. 1. Circulating testosterone levels in castrated rats were an order of magnitude below baseline levels of intact rats (Fig. 1, middle panel). When castrated rats were given a single subcutaneous injection of complexed testosterone (400 μg/kg body weight), the concentration in serum rose dramatically and began a decrease immediately (Fig. 1, middle panel) at a rate that appears to mimic an episode of an intact rat. Multiple administration of testosterone complex did not lead to any buildup of hormone in the animals. When castrated rats ($N = 3$) were given 30 daily injections of complexed testosterone at a much higher dose (1600 μg/kg body weight) than used in the behavior-physiology study, the circulatory testosterone on the day following the last administration was at typical castrate levels (Fig. 1, middle panel).

The principal experiment with young adult rats involved once-a-day supplementation of complexed testosterone by subcutaneous injection for 39 days. During days 37–39 behavior tests were performed, on the 40th day animals were killed, and physiology-related parameters were measured. Four groups of animals were supplemented: the dose–response study on castrates used daily means of 100, 300, or

Table I. Androgen-Sensitive Behaviors and Physiology of Adult Male Rats Given Daily Doses of a Water-Soluble Testosterone (T)*

	0 μg T/kg		100 μg T/kg		300 μg T/kg		800 μg T/kg		Intact + 300 μg T/kg	
	Castrate	Intact	Uniform	Rhythmic	Uniform	Rhythmic	Uniform	Rhythmic	Uniform	Rhythmic
Behavior										
Aggression										
Frequency	2 ± 1[a]	20 ± 1[d]	6 ± 1[a]	11 ± 1[b]	12 ± 1[b]	17 ± 2[c]	14 ± 1[c]	17 ± 1[c]	19 ± 1[c]	25 ± 2[e]
Sexual performance										
Latency intromission	>1800[a]	<5[e]	>1800[a]	>1800[a]	20 ± 2[c]	20 ± 1[c]	13 ± 1[d]	13 ± 1[d]	<5[e]	<5[e]
Latency ejaculation	>30[a]	7 ± 1[c]	>30[a]	>30[a]	26 ± 2[ab]	25 ± 2[ab]	22 ± 2[b]	21 ± 2[b]	7 ± 1[c]	5 ± 1[c]
Sexual motivation										
Proximity to estrous	505 ± 17[a]	720 ± 35[d]	479 ± 16[a]	518 ± 19[a]	634 ± 25[b]	650 ± 22[b]	641 ± 11[b]	665 ± 16[c]	670 ± 14[c]	732 ± 17[d]
Urine markings to estrous	3 ± 2[a]	90 ± 14[d]	7 ± 4[ab]	14 ± 6[b]	66 ± 15[c]	60 ± 10[c]	93 ± 11[d]	174 ± 24[f]	111 ± 19[e]	171 ± 25[f]
Physiology (weight)										
Body (g)	523 ± 5[b]	500 ± 5[a]	484 ± 6[a]	515 ± 9[b]	517 ± 3[b]	518 ± 8[b]	519 ± 4[b]	533 ± 6[c]	500 ± 6[a]	521 ± 4[b]
Organs										
Penis (mg)	89 ± 1[a]	105 ± 3[c]	93 ± 1[b]	105 ± 2[c]	103 ± 2[c]	102 ± 2[c]	106 ± 2[c]	104 ± 1[c]	107 ± 2[c]	123 ± 3[d]
Preputials (mg)	82 ± 2[a]	330 ± 5[e]	89 ± 2[a]	137 ± 7[b]	212 ± 6[c]	285 ± 7[d]	239 ± 6[c]	296 ± 10[d]	341 ± 6[e]	321 ± 7[e]
Seminar vesicle/ant. prostate (mg)	145 ± 3[a]	1018 ± 32[g]	200 ± 7[b]	247 ± 8[c]	330 ± 10[d]	378 ± 8[e]	380 ± 14[e]	438 ± 11[f]	1104 ± 31[h]	1163 ± 27[i]
Muscles										
Spongiosus, dorsal (mg)	107 ± 8[a]	287 ± 10[f]	127 ± 5[b]	135 ± 5[b]	182 ± 6[c]	221 ± 12[d]	253 ± 12[e]	292 ± 10[f]	314 ± 6[g]	359 ± 8[h]
Spongiosus, ventral (mg)	395 ± 18[a]	1114 ± 32[g]	438 ± 10[b]	463 ± 7[c]	644 ± 20[d]	720 ± 24[e]	811 ± 31[f]	811 ± 24[f]	1299 ± 27[h]	1409 ± 15[i]

* The terms "uniform" and "rhythmic" are defined under Results in the upper panel in Fig. 2. Units are defined under Materials and Methods. Six or more animals per group. Superscripts of different letters denote groups which differ significantly in Tukey's HSD ($P < 0.05$).

800 μg of testosterone/kg of body weight; and the fourth group was comprised of intact rats supplemented with 300 μg of testosterone/kg of body weight. However, within each dose level the administration of hormone was scheduled differently. In the uniform-pulse group the animals were injected with the same dose (given above) each day, whereas in the rhythmic-pulse group the same total of testosterone was used but the amount was divided into daily doses at a $\frac{1}{2}:\frac{1}{2}:2$ ratio for 36 days. On days 37–39 these animals also received testosterone in doses of the corresponding uniform pulse to ensure that circulatory levels of hormone in the respective groups were comparable when the behavior tests were conducted. These regimens are depicted in the upper panel in Fig. 2. In addition to the treated groups, a group of untreated castrates and sham-operated intact rats was maintained and used as controls.

In the behavior tests aggression such as pushing, sideways kicking, aggressive grooming and posturing, and attacks with biting between each subject and a target male was measured. Sexual performance was evaluated during interaction with an estrous female; sexual motivation was assessed by urinary markings around and the time spent in proximity to an inaccessible estrous versus a nonestrous female. Physiological parameters which were examined related principally to sexual functions. The results collected on young adult rats are given in Table I and are further summarized in the bar graph at the bottom in Fig. 2. Both physiology and behavior exhibited a dose–response relation to the amount of testosterone complex injected. Physiological indices [range of F values (1 or 4, 50 df) = 4.47–412.33, $P < 0.05$] were more affected by the supplementation than the behavior indices [range of F values (1 or 4, 50 df) = 3.64–338.06, $P < 0.05$]. Statistically significant differences of the groups with Tukey's HDS test ($P < 0.05$) are noted in Table I with superscript letters.

Although the results are not always consistent from the various measures, a clear pattern emerged among the groups. Animals receiving testosterone in rhythmic pulses experienced a more pronounced increase in androgen-sensitive behavior and physiology than animals receiving the same amount of testosterone complex in uniform pulses. At the highest dose (800 μg/kg body weight) administered in rhythmic pulses, the parameters measured on castrates approached those of intact rats. Overall body weight increased

Table II. Effects of Testerone Supplementation on Senescent Male Rats

	Group	
	Treated	Control
Sexual motivation		
Proximity to estrous ♀[a]		
Before regimen	13 ± 1	14 ± 2*
After regimen	12 ± 1	11 ± 1
Urinary markings to estrous ♀[a]		
Before regimen	44 ± 5*	39 ± 3*
After regimen**	60 ± 4	30 ± 3
General physiology		
Body weight (g)	520 ± 20	540 ± 10
Overall appearance	3 good/3 moderate/1 poor	2 good/4 moderate/1 deceased
Reproductive physiology		
Penis (mg/100 g body wt)	26 ± 1	24 ± 1
Testis (left)** (mg/100 g body wt)	308 ± 16	373 ± 12
Testis (right)** (mg/100 g body wt)	287 ± 16	358 ± 10
Epididymides		
Right—whole organ** (mg/100 g body wt)	141 ± 6	175 ± 11
Left—caput (% total wt)	44 ± 1	42 ± 2
Left—corpus (% total wt)	13 ± 1	12 ± 1
Left—caudal** (% total wt)	43 ± 4	47 ± 2
Seminal vesicles/coagulating gl. (mg/100 g body wt)	244 ± 5	244 ± 10
Ventral prostate** (mg/100 g body wt)	67 ± 5	53 ± 8
Preputials (mg/100 g body wt)	35 ± 2	38 ± 3
Muscles		
Abdominal** (mg/100 g body wt)	2300 ± 50	2090 ± 30
Spongiosus		
Dorsal (mg/100 g body wt)	62 ± 6	70 ± 2
Ventral (mg/100 g body wt)	209 ± 10	225 ± 8
Sperm		
Epididymis		
Right—whole organ** (count/g tissue)	467 ± 12 × 10^6	440 ± 15 × 10^6
Left—caput** (% total count)	38 ± 2	29 ± 2
Left—corpus (% total count)	6 ± 1	6 ± 1
Left—caudal** (% total count)	55 ± 2	65 ± 2

[a] Units are defined under Materials and Methods; seven and six animals in treated and control groups, respectively.
* Statistically significant difference ($P < 0.05$) within group.
** Statistically significant difference ($P < 0.05$) between the treated and the control group.

slightly and weight of muscles decreased upon castration. These effects on muscles were partially corrected by testosterone supplementation.

Comparisons of treated and untreated intact males proved interesting. Dosing intact males with 300 μg/kg body weight led to a "supraphysiological" response with increased sexual and aggressive behaviors, but only in the rhythmic pulse subgroup. Furthermore, the increase in muscle weight found in supplemented intact rats occurred without any forced exercise.

The effects of testosterone supplementation on senescent rats were measured when animals were 2 years old (i.e., at an age when in this colony only about half of the animals are still alive). Only uniform pulse daily supplementation of 400 μg testosterone/kg body weight for 60 days was tested. Behavior and physiological parameters of treated and untreated males were compared, and the results appear in Table II. Statistical analyses revealed within-group differences in behavior [range of $t(6 \text{ or } 5)$ values = 2.79–3.55, $P < 0.05$] and between-group differences in both behavior and physiology [range of $t(11)$ values = 2.72–3.32, $P < 0.05$]. Sexual motivation was improved notably with supplementation. Weight of testes decreased by approximately 20%, but ventral prostate weight increased by 25%. Supplementation led to an improvement in weight of skeletal muscle (10%), whereas body weight was slightly decreased. Examination of sperm counts revealed that supplementation by testosterone complex modified sperm reserves in the epididymis. There was a statistically significant increase in the total number of gametes [$t(11) = 2.3$, $P < 0.05$] in the supplemented group, and a significantly higher percentage of gametes was found in the caput region of the epididymis [$t(11) = 3.15$, $P < 0.05$]. These results suggest that renewed spermatogenesis had occurred.

DISCUSSION

In adult rats, the serum concentration of testosterone

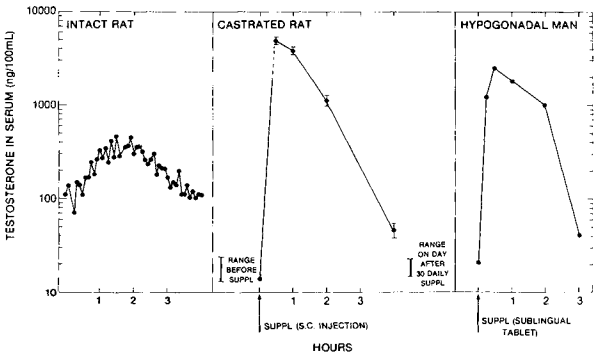

Fig. 1. Serum levels of testosterone. Left panel: during the natural episodic release in intact rats (adapted from Ref. 2). Middle panel: after subcutaneous injection of testosterone (400 μg/kg):2-hydroxypropyl- β-cyclodextrin complex into castrated rats. Right panel: after sublingual administration of a tablet containing testosterone (150 μg/kg):2-hydroxypropyl-β-cyclodextrin complex to a hypogonadal male (adapted from Ref. 12).

fluctuates during each day but remains at least an order of magnitude above that found in castrated animals or females. The pharmaceutical form used in this study elevated circulating levels of testosterone in castrates only for about a fifth of the day, whereas during the remainder of the day, the animals had levels typical of castrates. Yet this pulsatile supplementation improved physiological parameters of the reproductive system, weight of muscles, and behavior in a dose–response manner. These unexpected results were further strengthened by the findings that the rhythmic mode of administration, in which a relative high-amplitude dose was administered periodically, had better corrective effects than when the uniform mode was used, in which the same total amount of hormone was divided into equal doses. The conclusion seems to be that in order to maintain a number of qualities characteristic of maleness, periodical exposures to high levels of testosterone may be as important as the total amount of testosterone supplemented.

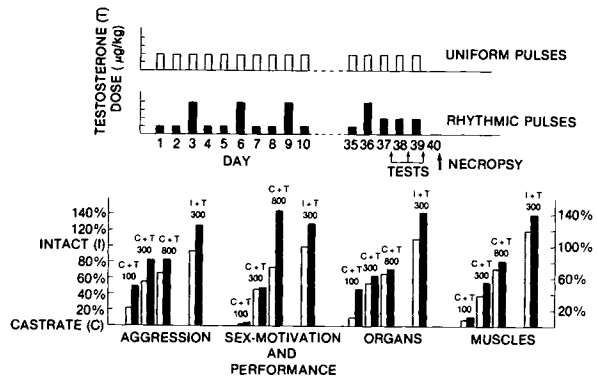

Fig. 2. The restoration of androgen-sensitive parameters after uniform (open bars) or rhythmic (filled bars) supplementation with testosterone:hydroxypropyl-β-cyclodextrin complex. C + T 100, castrated rats supplemented with 100 μg (average) of testosterone/kg of body weight per day; I + 300, intact rats supplemented with 300 μg (average) of testosterone/kg of body weight per day.

In senescence, androgens in rats are also decreased (16). The main endocrine change in aging mice is a decrease in the amplitude of episodal testosterone release (17). Thus, it seems that additional high-amplitude episodes by exogenous testosterone to gonadally intact old males may be optimal for correction. Indeed that approach, tested on senescent rats in this study, was effective; both behavior and physiological parameters of the supplemented group were improved compared to those of controls.

Administration of exogenous hormone has been shown to have untoward effects and for androgens these were recorded in both men and nonhuman males (6,24,25). When implants releasing constant amounts of testosterone were used in intact rats, the "lower" doses (about 400 μg/kg/day) led to azoospermia and reduction of testicular weight (up to 50%). Higher doses (4000 μg/kg/day) maintained spermatogenesis but doubled the weights of seminal vesicles and ventral prostate (8). The considerable capacity of the organism to metabolize testosterone when this hormone is released from implants in constant amounts undoubtedly plays a role in this type of supplementation (26). The dosage used in the present study of testosterone pulses was generally lower (100–800 μg/kg/day), and while corrections in behavior and physiology were obtained, the untoward effects (e.g., on prostate growth) were smaller. Furthermore, pulsatile administration of testosterone in small doses appeared to stimulate spermatogenesis.

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