Effects of Acute Stanozolol Treatment on Puberty in Female Rats

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ABSTRACT

The effects of anabolic-androgenic steroid (AAS) abuse on the onset of puberty in female adolescents are largely unknown. This study assessed the acute effects of one AAS, stanozolol, on pubertal onset in the female rat. A single injection of stanozolol (5 mg/kg) on Postnatal Day (PN) 21 advanced vaginal opening but did not alter the onset of vaginal estrus. Higher doses of stanozolol treatment (10 and 25 mg/kg) also advanced vaginal opening but had no effect on vaginal estrus. The advancement of vaginal opening by stanozolol (5 mg/kg) was prevented by the concomitant administration of the pure antiestrogen ICI 182,780 (1 mg/kg) on PN20–22. Administration of the androgen receptor antagonist flutamide (10 mg/kg twice daily) on PN20–22 had no effect on the advancement of vaginal opening by stanozolol. Stanozolol treatment also advanced vaginal opening in ovariectomized rats. Perivaginal injections of a low dose of stanozolol (0.05 mg) on PN21 and PN23 also advanced vaginal opening. These results suggest that stanozolol is acting directly at estrogen receptors in the vaginal epithelium to advance vaginal opening and that prepubertal stanozolol treatment does not induce true precocious puberty.

INTRODUCTION

Anabolic-androgenic steroids (AAS) are synthetic derivatives of testosterone that have been abused by athletes in an attempt to improve athletic performance [1]. In contrast to AAS abuse by adolescent males, which has remained at a steady level since 1991, AAS abuse by adolescent females has actually increased during this same time period [2]. AAS abuse by females has been associated with a number of adverse effects, including menstrual abnormalities and reproductive dysfunction [3–5]. Studies have been focused on the adverse effects of AAS abuse in adult females [3, 6]; however, the physiological effects of AAS abuse by adolescent females are largely unknown, and there is concern that some of the effects may be permanent [4].

In rat models, there is overwhelming evidence that puberty can be advanced by estrogen and aromatizable androgens [7, 8]. In contrast to the advancement of puberty by estrogen and aromatizable androgens, treatments with non-aromatizable androgens delay the onset of puberty [9, 10]. Thirty-day treatment with one AAS, stanozolol, has differential effects on pubertal endpoints in female rats. Chronic stanozolol treatment advanced the day of vaginal opening (VO) but inhibited vaginal cyclicity [11]. Interpretation of the effects of chronic stanozolol administration on the onset of puberty, as defined by VO and the onset of estrous cyclicity, is confounded by the alteration of the feedback loops of the hypothalamus-pituitary-gonadal (HPG) axis coinciding with the time when puberty normally occurs. The present study was conducted to investigate the effects and mechanism(s) of action of the acute administration of one AAS, stanozolol, in prepubertal rats.

MATERIALS AND METHODS

Animals

Timed-pregnancy Long-Evans rats used in these experiments were obtained from Harlan Sprague-Dawley (Indianapolis, IN) and Charles River Laboratories (Wilmington, MA). Rats from Harlan were used until sialodacryoadenitis virus (SDAV) appeared in the Harlan stock, at which point the rats were then obtained from Charles River at the recommendation of our veterinary staff. The stock used for each experiment is noted. Investigations were conducted in accordance with the Guide for the Care and Use of Laboratory Animals.

Procedures and Analysis

The following drugs were used: stanozolol (17α-methyl-5α-androstan-17β-olo(3,2-c)pyrazole; Sigma Chemical Co., St. Louis, MO), flutamide (2-methyl-N-[4-nitro-3-(trifluoromethyl)-phenyl]propanamide; Sigma), ICI 182,780 (7α-[9-[(4,4,5,5,5-pentafluoropentyl)sulphinyl]monyl]-estra-1,3,5(10)-triene-3,17β-diol; Tocris Cookson, Ballwin, MO), medroxyprogesterone (17β-hydroxy-17-methyl-androsta-1,4-dien-3-one; Sigma), and dihydrotestosterone propionate (5α-androstan-17β-ol-3-one propionate; Steraloids, Newport, RI). Drugs were administered by s.c. injection in a sesame oil vehicle, with the exception of flutamide, which was administered in a 10% ethanol/propylene glycol vehicle. Drugs were coded, so the experimenter was blind to the treatment conditions.

The female pups were monitored daily for VO between approximately 0800 and 1000 h. Once VO had occurred, the vaginal cytology of the rats was monitored daily until PN65 (see exception for experiment 3A). The criterion for vaginal estrus was vaginal cytology that contained epithelial cells and nucleated epithelial cells [12]. In experiment
3A, ovariectomy was carried out under sodium methohexital (Brevital, 50 mg/kg; Henry Schein, Port Washington, NY) anesthesia. Although body weight was monitored throughout the course of the experiments, body weight data are not presented because there were no differences between treatment groups in any of the experiments.

All data sets, with the exception of the data from experiment 1B, met the assumption of a normal distribution and homogeneity of variance. The dependent measures, such as day of VO and day of first vaginal estrus, were evaluated by one-way analysis of variance, and post hoc analysis was performed with a Newman-Keuls test. The data from experiment 1B did not exhibit homogeneous variance and were thus subjected to nonparametric analysis (Kruskal-Wallis followed by Mann-Whitney U-test). Results are expressed as means ± SEM for each group. Differences were considered significant at \( P < 0.05 \).

**Experiment 1: Acute Effects of Stanozolol Treatment on Aspects of Pubertal Onset**

In experiment 1A, at 0900 h on PN21, female pups from Harlan were weaned, weighed, randomly assigned to one of two groups (\( n = 6 \) pups/group), and given a single injection of either stanozolol (5 mg/kg) or the sesame oil vehicle (1 ml/kg) \[13\]. PN21 was chosen as the age to treat the pups based on previous research \[11, 14\]. Experiment 1B was conducted to establish the dose response characteristics of a single injection of stanozolol. At 0900 h on PN21, female pups from Charles River were weaned, weighed, randomly assigned to one of five groups (\( n = 7 \) or 8 pups/group), and given a single injection of 0, 1, 5, 10, or 25 mg/kg stanozolol in a sesame oil vehicle.

**Experiment 2: Stanozolol Action at Androgen and Estrogen Receptors**

Experiment 2A was conducted following the same design as experiment 1, with the addition of groups receiving hormone receptor antagonists. Rats from Harlan received 5 mg/kg stanozolol or the sesame oil vehicle in combination with 1) the nonsteroidal androgen receptor (AR) antagonist flutamide (10 mg/kg twice daily), 2) the pure estrogen receptor (ER) antagonist ICI 182,780 (ICI, 1 mg/kg), or 3) the oil vehicle (1 ml/kg). The groups were as follows: stanozolol/ICI (\( n = 5 \) for oil/ICI), stanozolol/oil (\( n = 5 \) for oil/oil, 35.0 ± 6.2, \( P < 0.05 \)).

**Experiment 2B** was conducted following the same design as experiment 1A, with the addition of groups receiving hormone receptor antagonists. Rats from Harlan were weaned, weighed, randomly assigned to one of five groups (\( n = 5 \) pups/group), and given a single injection of 0, 1, 5, 10, or 25 mg/kg stanozolol in a sesame oil vehicle.

Results are expressed as means ± SEM for each group. Differences were considered significant at \( P < 0.05 \).

**Experiment 3: Stanozolol Action at Central Versus Peripheral Targets**

Two experiments were conducted to examine the site of action of stanozolol on VO. In experiment 3A, 16 female rats from Charles River were ovarioctomized on PN20. One day later these rats received a single injection of either stanozolol (5 mg/kg, \( n = 8 \)) or oil (1 ml/kg, \( n = 8 \)). Estrous cyclicity was not monitored for this experiment. In experiment 3B, female rats were given perivaginal injections of 0.05 mg stanozolol in 0.01 ml oil (\( n = 6 \)) or the oil vehicle (\( n = 6 \)) on PN21 and PN23. The time course and procedure were similar to that used previously \[14\], where it was shown that perivaginal s.c. injections of 0.01 mg testosterone advanced VO.

**RESULTS**

**Acute Effects of Stanozolol Treatment on Aspects of Pubertal Onset**

Vaginal opening vs. vaginal estrus. There was a dissociation between the effects of stanozolol on the day of VO and on the day of first vaginal estrus. As shown in Figure 1A, the day of VO was advanced significantly in the stanozolol group as compared with the oil group (\( F(1, 10) = 6.2, P < 0.05 \)). In contrast to the effects of stanozolol on VO, the treatment groups did not differ for the day of first vaginal estrus. Furthermore, no long-term effects of a single injection of stanozolol on estrous cyclicity (including cycle length) were observed. During the 14 days after the first vaginal estrus, there were no differences in the number of days of vaginal estrus between the stanozolol and oil groups (Fig. 1B).

**Dose response.** As shown in Figure 2, dose of stanozolol affected day of VO (\( H = 14.5, P < 0.05 \)). The day of VO for the 10 and 25 mg/kg stanozolol groups was significantly different than that for the oil group (\( P < 0.05 \)). Although there was a trend toward advanced VO in the 5 mg/kg stanozolol group, the effect was not significant. In subsequent experiments using rats from Charles River, 5 mg/kg stanozolol was sufficient to advance day of VO (experiments 2B and 3A). As seen in experiment 1, stanozolol (1–25 mg/kg) had no significant effect on the day of first vaginal estrus.

**Stanozolol Acts via the ER to Advance VO**

Stanozolol appears to advance VO via an ER-dependent mechanism. As shown in Figure 3, the day of VO was advanced significantly in the stanozolol/oil group as compared with the oil/oil group (\( F(3, 45) = 12.6, P < 0.05 \)). ICI administration blocked the advancement of VO by stanozolol so that the day of VO of the stanozolol/ICI group was not significantly different from that of the oil/oil group. In contrast, AR blockade had no effect on the advancement of VO by stanozolol. Stanozolol advanced VO in rats receiving flutamide relative to the oil/oil group (\( P < 0.05 \)). The day of VO of the stanozolol/flutamide group was not significantly different from that of the stanozolol/oil group. As seen in experiment 1, treatment conditions did not have a significant effect on the day of first vaginal estrus.

In experiment 2B, administration of another synthetic androgen had an effect on day of VO. As shown in Figure...
FIG. 1. Experiment 1A: pubertal events of rats that received 5 mg/kg stanozolol or oil on PN21. A) Postnatal day of VO and of first vaginal estrus of rats that received oil (n = 12 or 13 pups/group). The stanozolol or oil was administered on PN21, and the data points are means + SEM. B) Number of days of vaginal estrus for the 14 days following the first vaginal estrus. *Significantly different from oil group (P < 0.05).

FIG. 2. Experiment 1B: postnatal day of VO and of first vaginal estrus of rats that received oil or 1, 5, 10, or 25 mg/kg stanozolol (n = 7 or 8 pups/group) on PN21. Data points are means + SEM. *Significantly different from oil group (P < 0.05).

FIG. 3. Experiment 2A: postnatal day of VO and of first vaginal estrus of rats that received oil/oil, stanozolol/oil, stanozolol/ICI, or stanozolol/flutamide (n = 12 or 13 pups/group). The stanozolol or oil was administered on PN21, and the ICI, flutamide, and oil were administered on PN21–23. Data points are means + SEM. *Significantly different from oil/oil group (P < 0.05).

FIG. 4. Experiment 2B: postnatal day of VO and of first vaginal estrus of rats that received 5 mg/kg stanozolol, 7.5 mg/kg methandrostenolone, or oil (n = 10 or 11 pups/group) on PN21. Data points are means + SEM. *Significantly different from oil group (P < 0.05).

4, in agreement with the results from the previous experiments, the day of VO was advanced significantly in the stanozolol group as compared with the oil group (P < 0.05). In addition, the day of VO was also advanced significantly in the methandrostenolone group as compared with the oil group (P < 0.05). As before, the treatment conditions had no effect on the day of first vaginal estrus. As shown in Figure 5, there were no differences in either the day of VO or the day of first vaginal estrus between the group receiving the nonaromatizable androgen DHTP and the group that received the vehicle.

Stanozolol Acts in the Periphery, not Centrally, to Advance VO

Experiment 3A tested the effects of stanozolol on VO in ovariectomized rats. As shown in Figure 6, VO was advanced in the ovariectomized rats that received stanozolol as compared with the ovariectomized rats that received the oil vehicle (F(1, 14) = 28.0, P < 0.05), indicating that the
ovaries are not necessary for the advancement of VO by stanozolol. As of PN60, in agreement with previous published studies [18, 19], a portion of the ovariectomized/oil rats exhibited VO (five of eight rats). The three rats that did not exhibit VO during the experiment were assigned a value of PN60 in the analysis.

In experiment 3B, rats were injected perivaginally with a low dose of stanozolol. As illustrated in Figure 7, VO was advanced in the group that received perivaginal injections of stanozolol as compared with the group that received perivaginal injections of the oil vehicle. As in the previous experiments, there was no difference in the day of first vaginal estrus between the stanozolol group and the vehicle group.

DISCUSSION

Effects of Acute Stanozolol Treatment on the Onset of Puberty

A single injection of stanozolol advances VO when administered to prepubertal rats. In contrast to effects on VO, acute stanozolol treatment on PN21 did not alter the onset of estrous cyclicity. Both the day of first vaginal estrus and number of days of vaginal estrus that occurred during the 14 days following the first vaginal estrus were similar for rats that received either stanozolol or the oil vehicle. The latent effects of stanozolol on VO can be related to the effects of estrogen administration during a similar time frame. Estrogen administration (estradiol benzoate, 0.05 μg/100 g body weight on PN26–30) advanced VO [7]. Presumably administration of estrogen or stanozolol sets into motion a cascade of physiological events resulting in VO. In contrast to the effects of stanozolol, however, estrogen also advances the onset of vaginal estrus [7]. Thus, stanozolol treatment does not induce true precocious puberty.

There were apparent differences in the onset of puberty between the two source stocks of rats used in these three experiments. Although statistical comparisons between the two control groups were not conducted, the control rats from Charles River appeared to achieve VO and first vaginal estrus at a younger age than did the control rats from Harlan. The ranges of means for the control rats from Charles River were 28.7–31.3 for the day of VO and 30.7–35.6 for the day of first vaginal estrus. For the control rats from Harlan, the ranges were 34.5–37.5 for day of VO and...
35.3–39.3 for day of first vaginal estrus. The first ovulation in rats occurs over a range of days that differs between different laboratory stocks [8]. Despite the apparent differences between the stocks used in our experiments, stanozolol advanced VO in both stocks across all three experiments.

**Mechanism and Site of Action**

Our results suggest that stanozolol is acting at the peripheral ER to advance VO. Specifically, treatment with the ER antagonist ICI prevented the advancement of VO by stanozolol. In pilot experiments, we determined that administration of ICI alone on PN21 had no effect on VO. Because VO is an estrogen-dependent event, administration of the pure antiestrogen ICI would be predicted to delay VO. No studies have been conducted, however, administering ICI at peripubertal time points. The observation that the administration of stanozolol on PN21 advances the day of VO whereas ICI administration on the same day has no effect on day of VO may appear inconsistent with previous studies indicating that VO is an estrogen-dependent event [8] and the possibility that stanozolol advances VO via actions at the ER. However, a number of possibilities exist that may account for this apparent disparity. For example, the critical time during which an estrogenic stimulation is required may occur later than PN21. Stanozolol, which is designed for enhanced metabolic half-life [1], may still be active at later ages, whereas the actions of ICI may be limited to the time period just after injection. If this is the case, then injection of ICI at later ages (PN35+) would be predicted to block VO. The possibility remains to be tested, however, because of the vendor's tight restrictions on the availability of ICI (100 mg per institution per year).

The data collected using ICI alone does not provide sufficient evidence to conclude that stanozolol is acting directly at the vaginal ER to advance VO [20]. Even though ICI, which is thought unable to cross the blood-brain barrier [17], blocks the advancement of VO by stanozolol, the final common pathway for either direct or indirect actions of stanozolol on VO would be the activation of ER at the vagina, which would be blocked by ICI in either case. More compelling support for the hypothesis that stanozolol is acting in the periphery to advance VO is that if stanozolol were acting centrally to advance VO, e.g., by mimicking estrogen and stimulating LH release [21], then both VO and estrous cyclicity would be advanced and true precocious puberty would result. The data from the present experiments indicate that this is not happening. Stanozolol treatment only advanced VO and did not have any effect on the onset of vaginal estrus. In the present experiments, we measured vaginal estrus rather than ovulation to follow the cycle of the rats for the 2 wk following the first vaginal estrus and to observe potential long-term effects of stanozolol on estrous cyclicity. Further experiments need to be conducted to determine the effects of stanozolol on ovulation.

Additional data in the present experiments support the hypothesis that stanozolol is acting directly at the vaginal epithelium to advance VO. For example, stanozolol advanced VO in ovariectomized rats. If stanozolol were acting via a central mechanism to advance VO, then it would most likely act indirectly through the ovaries to trigger VO, and yet stanozolol advanced VO in the absence of ovaries. This result is similar to those of previous experiments, where the treatment of ovariectomized pubertal rats with aromatizable androgens advanced VO, ruling out an effect dependent on the function of the ovary [22–24]. In other experiments conducted in our laboratory, systemic administration of stanozolol induced central effects, as evidenced by changes in behavior [25, 26]. We hypothesize that the lack of central action of stanozolol in the present study is due to the limited time course (a single injection) of stanozolol administration, the weak affinity of stanozolol for the ER, or some combination of these factors. Moreover, these same factors are likely to contribute to the dissociation between the effects of estrogen and stanozolol on VO versus vaginal estrus. The results from localized injections of stanozolol provide additional support for the hypothesis that stanozolol is acting at the vaginal epithelium to advance VO. Perivaginal injections of low doses of stanozolol advanced VO, whereas the same low dose of stanozolol had no effect on VO when administered systemically.

Stanozolol does not appear to be acting at the AR to advance VO; AR blockade by flutamide had no effect on the advancement of VO by stanozolol. The dose of flutamide used was sufficient to block the inhibition of estrogen-induced receptivity by stanozolol, suggesting that the dose was adequate [15]. There is no evidence in the literature that the AR is involved in the advancement of VO. Contrary to the actions of estrogen and aromatizable androgens, nonaromatizable androgens either have no effect on the onset of puberty or delay it [9, 10]. Taken together, the results of the present experiments suggest that stanozolol may be acting at the vaginal ER to advance VO. This result is unexpected given that stanozolol cannot be aromatized [27]. The data presented here provide only indirect evidence that stanozolol is acting at the ER. Additional analyses of binding of stanozolol to the ER or evaluation of stanozolol action in ER knockout mice are essential to confirm this hypothesis. We plan to test stanozolol in estrogen-response element assays to clarify whether stanozolol is capable of binding to and activating the ER, as has been shown for other synthetic compounds and xenoestrogens [28, 29]. Such studies will provide a clearer picture of the mechanisms underlying the acute effects of AAS on the maturation of reproductive function. These findings will help us understand the influence of AAS use during adolescence on female reproductive health.

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