EFFECTS OF NEONATALLY INJECTED NON-ESTERIFIED TESTOSTERONE ON REPRODUCTIVE FUNCTIONS IN FEMALE RATS

By
T. Alklint and A. Norgren

ABSTRACT
The effect of 1.5 mg testosterone (T) given to neonatal rats of various ages was compared with that of 1.5 mg testosterone propionate (Tp) injected into 5 day old rats.

Androgenization of the rats was obtained:
1) with Tp in 100 per cent,
2) with T on day 2 in 79 per cent,
3) with T on day 5 in 50 per cent,
4) with T on day 10 in 18 per cent,
5) with a double dose of T on day 5 in 61 per cent,
6) with T on day 5 and 10 in 100 per cent.

The results emphasize the importance of the duration of action of the preparation given. The possibility is considered that a minimal exposure time of more than a few hours is required to produce androgenization of 5 day old female rats.

The length of the neonatal period during which androgens have organizational effects on the brain of rats (the "critical period of heightened sensitivity") has been assessed to be 5–10 days (Harris 1964; Jacobsohn 1965; Gorski 1966; Barraclough 1967; Arai & Gorski 1968a,b; Gorski 1968). Subsequent modifications of the reproductive functions seem to depend on the dose of the hormone given, the route of administration, and the age of the rat at the time of injection. Ovulations may fail to occur and constant vaginal cornification may be present from puberty onwards ("early androgen syndrome", EAS), or occasionally corpora lutea may be formed for a variable period of time after
puberty but before the mechanisms involved in the control of ovulation are permanently altered (»delayed androgen syndrome«, DAS).

The androgen mainly used was the long-acting preparation testosterone propionate in oil, and little attention has been paid to other testosterone preparations with less prolonged effects. Hence a delineation of the critical period for the production of the androgen syndrome is difficult since the duration of action of the preparations in the newborn rat is not known with certainty. It may be rather long: in spayed rats injected with 1.5 mg testosterone propionate in oil subcutaneously on day five after birth, mammary changes indicating a response to androgen were seen for approximately 15 days (Jacobsohn & Norgren 1965). The same dose (1.5 mg) of testosterone in propylene glycol given on day five was without any effect on the mammary gland (Jacobsohn & Norgren, unpublished). It should be mentioned that Kincel et al. (1965) estimated the minimum effective dose of testosterone propionate in oil (given on day 5) required for androgenization in rats to be 10 µg, as against 1000 µg for testosterone.

The present paper deals with the development of the »androgen syndrome« in neonatal rats injected with testosterone or testosterone propionate dissolved in propylene glycol at different ages.

**EXPERIMENTAL**

Rats of an inbred strain kept at the Institute were used. The litters were kept in separate cages. All litters were reduced to six three days after birth, usually by removing males. A pelleted diet and water were freely available.

Testosterone in propylene glycol (1.5 mg in 0.03 ml) was given subcutaneously: *as a single injection* 1) within 48 hours after birth, 2) at 5 days of age*, 3) at 10 days of age, or *as two injections* (1.5 mg each) a) on day 5 (given simultaneously, but at separate sites in order to obtain a similar rate of absorption in the different groups of rats), b) on day 5 and 10.

Testosterone propionate in propylene glycol (1.5 mg in 0.03 ml) was given on day 5 to another group of rats.

The different treatments were randomly distributed among the littermates. One or two rats from each litter were kept as uninjected controls.

Ten days after birth and thereafter the vaginal orifice was inspected once or twice daily. Vaginal smears were taken when the rats were 21 days old (i.e. at weaning) or when the vaginal orifice opened. Smears were taken daily until the rats were three months old. The smears were stained with methylene blue. The day on which the smears showed cornified cells only (no mucin or leucocytes present) was regarded as the day of first oestrus. When the rats were three months old, or more, they were placed together with a male for 21 days in order to test their fertility.

* The day after birth was regarded as day one of the rats' life.

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RESULTS AND COMMENTS

The results are described and comments made on according to the treatment given (Table 1).

Group 1. Uninjected controls (60 rats)

In the group of uninjected control rats the age at the first vaginal oestrus

<table>
<thead>
<tr>
<th>Exp.</th>
<th>No. of rats</th>
<th>Age (days) at:</th>
<th>Constant oestrus</th>
<th>Fertility</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>vaginal opening</td>
<td>first oestrus</td>
<td></td>
</tr>
<tr>
<td>Group 1</td>
<td>60</td>
<td>36 ± 0.5</td>
<td>38 ± 0.6</td>
<td>0</td>
</tr>
<tr>
<td>(Untreated controls)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 2</td>
<td>12</td>
<td>12 ± 0.1</td>
<td>37 ± 0.8</td>
<td>12</td>
</tr>
<tr>
<td>(Tp, 1.5 mg on day 5)</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>Group 3</td>
<td>19</td>
<td>33 ± 0.8</td>
<td>35 ± 0.8</td>
<td>15</td>
</tr>
<tr>
<td>(T, 1.5 mg within 48 h)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Group 4</td>
<td>10</td>
<td>12 ± 0.2</td>
<td>32 ± 0.8</td>
<td>5</td>
</tr>
<tr>
<td>(T, 1.5 mg on day 5)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 5</td>
<td>11</td>
<td>17 ± 0.1</td>
<td>37 ± 1.0</td>
<td>2</td>
</tr>
<tr>
<td>(T, 1.5 mg on day 10)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 6</td>
<td>18</td>
<td>18 ± 1.4</td>
<td>34 ± 0.9</td>
<td>11</td>
</tr>
<tr>
<td>(T, 1.5 mg twice on day 5)</td>
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<td></td>
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</tr>
<tr>
<td>Group 7</td>
<td>11</td>
<td>12 ± 0.2</td>
<td>34 ± 0.8</td>
<td>11</td>
</tr>
<tr>
<td>(T, 1.5 mg on days 5 and 10)</td>
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</table>

1. Number of rats with constant vaginal oestrus at 90 days of age.
2. Number of fertile rats.
3. Mean ± standard error of the mean.
4. Four rats died before the test of fertility was completed.
5. One rat died before the test of fertility was completed.
was 38 ± 0.6\(^*\) days. In several rats a vaginal opening was observed a few days before the first vaginal oestrus; the oestrous cycles were usually regular. Fifty-two of 56 rats (93\%) tested became pregnant. Four rats died before the test for fertility was completed.

**Group 2. Testosterone propionate (Tp) 1.5 mg on day 5 (12 rats)**

The mean age at vaginal opening of the present group of rats was 12 ± 0.1 days. The mean age at first vaginal oestrus was 37 ± 0.8 days. Two rats presented a few irregular oestrous cycles during the experiment, but by 90 days of age a persistent vaginal cornification was established in all the rats, and none of them became pregnant.

In the present group of rats the age at vaginal opening was less (12 days) than that of the control rats (36 days, \(P < 0.001\)). The mean age at first vaginal oestrus was only a few days less than that of the controls (Table 1). The effects of Tp in propylene glycol was not significantly different from that obtained concomitantly from 7 rats (not included in the present material) injected on day 5 after birth with 1.5 mg of a commercial preparation of Tp in oil (Perandren\(^®\), Ciba).

**Group 3. Testosterone (T) 1.5 mg within 48 hours after birth (19 rats)**

The age at vaginal opening varied between 26 and 37 days, the mean being 33 ± 0.8 (19) days. The age at the appearance of the first vaginal oestrus varied considerably, the mean being 35 ± 0.8 days. The opening of the vagina to the exterior was abnormally narrow in most rats and was situated near to the urethra. By 90 days of age a persistent vaginal cornification was found in 15 rats (79\%). Four of the 15 rats had occasional vaginal oestrous cycles before the smears showed persistent cornification. Three rats (16\%) delivered young.

With the treatment used a high percentage of the rats were »androgenized«: the age at the first vaginal oestrus was less (\(P < 0.02\)) than that of the un-injected control rats, and the majority of rats presented a persistent vaginal cornification by 90 days of age. However, the effect on later reproductive functions of 1.5 mg T given within 48 hours after birth was less than that produced by 1.5 mg Tp given on day 5 (Group 2). The morphological abnormalities of the vaginal orifice were similar to those described by Tramezzani et al. (1963), who injected with Tp in oil (200 \(\mu\)g).

**Group 4. Testosterone 1.5 mg on day 5 (10 rats)**

The age at vaginal opening was 12 ± 0.2 days and the age at first vaginal oestrus was 32 ± 0.8 days. Five rats (50\%) had persistent vaginal cornification

\* Mean ± standard error of the mean.
by 90 days of age. Initially, four of them had irregular vaginal oestrous cycles. Three of the nine rats (33%) delivered young. One rat died during the experiment.

The age at vaginal opening and first vaginal oestrus was less \( P < 0.001 \) than that observed in untreated control rats, and vaginal opening occurred earlier than in rats injected shortly after birth \( P < 0.05 \); Group 3. In this respect 1.5 mg of T was more effective on day 5 than within 48 hours after birth, but with regard to later reproductive functions T was less effective on day 5. A comparison with Group 2 shows that the 1.5 mg dose of T was considerably less effective than the same dose of Tp given to rats of the same age.

**Group 5. Testosterone 1.5 mg on day 10 (11 rats)**

The mean ages at vaginal opening and first vaginal oestrus were 17 ± 0.1 and 37 ± 1.0 days, respectively. The vaginal smears showed fairly regular cycles in 9 rats. In the remaining two rats (18%) vaginal cornification was apparent by 90 days of age, though occasional cycles were seen during the period of observation. Nine of the rats (82%) became pregnant.

As found by other workers studying rats injected with Tp (see Barraclough 1967), in the present study with T the incidence of persistent abnormalities of reproductive functions decreased with increasing age of the rats at the time of injection. Thus, the percentages of rats showing persistent vaginal cornification from puberty onwards were 58, 10 and 0, in rats injected with T within 48 hours after birth, on day 5, and on day 10, respectively, while, by 90 days of age, persistent vaginal oestrus was found in 79, 50 and 18 per cent, respectively. The percentages of fertile rats were 16, 33 and 82, respectively. However, the results obtained with a single injection of T never approached those obtained with a single injection of Tp given on day 5 (Group 2).

**Group 6. Testosterone 3.0 mg on day 5 (18 rats)**

In the present group vaginal opening and first vaginal oestrus occurred at 18 ± 1.4 and 34 ± 0.9 days of the rats' age, respectively. Persistent vaginal oestrus was observed in 8 rats from puberty onwards. After a few cycles initially, persistent vaginal cornification developed in another three rats. Thus, by 90 days of age persistent vaginal cornification was present in 11 rats (61%). Four rats (22%) delivered young.

Because of the limited effect caused by 1.5 mg T given on day 5 (Group 4), the dose of T was doubled. Compared with that of Group 4, given 1.5 mg T, the effect of T in the present group on the later reproductive functions of the rats was only slightly enhanced, as indicated by a higher percentage of rats presenting persistent vaginal cornification and a lower frequency of fertile rats.
The unexpected finding that the mean age at vaginal opening of the present group was 18 days as compared with 12 days in Group 5 (a single injection of 1.5 mg T on day 5) cannot be explained. It should be mentioned, that a tiny vaginal opening may have been overlooked.

**Group 7. Testosterone 1.5 mg on day 5 and on day 10 (11 rats)**

The modifications of the reproductive functions of the rats in the present group were distinct. Vaginal opening occurred at 12 ± 0.2 days of age, and the age at first vaginal oestrus was 34 ± 0.8 days. Persistent vaginal cornification from puberty onwards was seen in 8 rats. The remaining 3 rats initially had a few cycles, after which a persistent vaginal cornification was recorded. One rat died before the test of fertility was completed. None of the 10 surviving rats delivered young.

As found in the present group the effect of two doses of T given with an interval of 5 days was the same as that obtained from Group 2 injected once with 1.5 mg Tp.

**DISCUSSION**

Testosterone propionate (Tp) in oil is a potent agent in the induction of permanent disturbances of the reproductive functions in female rats. Single doses of 10–30 µg injected neonatally are sufficient to make a high percentage of the rats sterile for the remainder of their lives (e.g. Kincl et al. 1965; Barraclough 1967; Gorski 1968). The dose of Tp (1.5 mg) used in the present study should be considered high and in view of the morphological abnormalities which occurred in the vagina of the present rats injected with 1.5 mg testosterone (T) within 48 hours after birth, the dose of T used would also seem to be high. Although Tp and T were both dissolved in propylene glycol and injected when the rats were 5 days old, their effects on later reproductive functions were markedly different. The result agrees with that of Kincl et al. (1965) who found that the minimum effective dose of T necessary to androgenize newborn rats was 1000 µg compared with 10 µg of Tp; the vehicle used was oil.

Since in the present study 1) the type of vehicle used did not seem to be of major importance and 2) the doses administered were high, interest becomes focused on the duration of action of the hormone preparations, which is known to be long in the case of Tp and short in the case of T (Miescher et al. 1936; Parkes 1936; Jacobsohn & Norgren 1965, and unpublished observations). That the effect produced by 1.5 mg T given on day 5 was only slight was probably due to the short duration of action of this hormone preparation. For the same reason it seems likely that 3 mg T given on day 5 was hardly more effective than 1.5 mg T on day 5. The effect of T on the reproductive functions
increased markedly when 1.5 mg T was given both on day 5 and 10. The finding that this treatment was almost as effective as that produced by testosterone propionate, given once to 5 day old rats, emphasize the importance of the duration of action of the preparation given.

The present observations would seem to indicate that the minimal exposure time to androgen required to induce permanent alterations in the reproductive functions of the female rat is longer than the 6–12 hour period suggested by Arai & Gorski (1968a,b). Arai and Gorski, however, used another approach.

SUMMARY

The effect of testosterone (T) given to neonatal rats of various ages was compared with that of testosterone-propionate (Tp) injected into 5 day old rats.

Androgenization of the rats was obtained 1) with Tp in 100 per cent, 2) with T on day 2 in 79 per cent, 3) with T on day 5 in 50 per cent, 4) with T on day 10 in 18 per cent, 5) with a double dose of T on day 5 in 61 per cent, 6) with T on days 5 and 10 in 100 per cent of animals. The results emphasize the importance of the duration of action of the preparation given. The possibility is considered that a minimal exposure time of more than a few hours is required to produce androgenization in 5 day old female rats.

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