Prolonged inhibition of normal ovarian cycles in the rat and cynomolgus monkeys following a single s.c. injection of danazol*

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In castrated male rats, a single s.c. injection of danazol has been shown to result in an inordinately prolonged inhibition of serum luteinizing hormone (LH) and follicle stimulating hormone (FSH) concentrations. In the present study, we have examined whether the same and similar routes of administration suppresses ovarian function in normally cycling rats and cynomolgus monkeys. Normally cycling female rats received danazol as a single administration either orally, i.m. or s.c. and a separate group also received danazol in silastic capsules. The duration of the dioestrous interval until the next oestrous smear was followed daily and cycle lengths were compared with vehicle-treated groups. Six normally cycling cynomolgus monkeys were followed by daily observation and blood sampling at 2–3 day intervals. After one normal cycle, danazol (200 mg/kg) was administered as a single s.c. injection. Monkeys were followed until the next menses and one cycle thereafter and blood samples were assayed for oestradiol, progesterone and bioactive LH. Oestrous cycle length in vehicle-treated control rats was 4.7 days. A single administration of danazol s.c. at the higher dose prolonged the dioestrous interval to 31.3 days (P <0.001) and a similar prolongation was observed with this high dose when administered i.m. (27.7 days; P <0.001). In normally cycling monkeys, the menstrual cycle length was 30.2 days, but following a single danazol administration, the mean duration to the next menses was prolonged to 117.5 days (P <0.001). In five out of six monkeys, there was a decrease in LH and an absence of normal oestradiol and progesterone patterns. After this prolonged hiatus, a subsequent menstrual cycle was normal in length and endocrine pattern. A single s.c. administration of danazol resulted in a prolonged suppression of ovarian cyclicity in both normally cycling rats and cynomolgus monkeys.

Key words: danazol/menstrual cycle/monkeys/oestrous cycle/ rats

Introduction

In an earlier report, we demonstrated the unexpected finding that a single s.c. injection of danazol to castrated male rats resulted in a prolonged (>25 days) suppression of serum concentrations of both luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Castracane et al., 1994). This action represents an interference with the negative feedback loop of gonadotrophin regulation in the castrated male rat. In the present study, we have examined this and other routes of administration of danazol in the normally cycling female rat and effects of a single s.c. administration in the normally cycling cynomolgus monkey. Any effect on the ovarian cycle in either species would represent an interference with both the negative and positive feedback effects during the cycle and it would be closer to any intended clinical uses for danazol. The results of this study complement those of our earlier study and demonstrate a prolonged inhibition of ovarian function following a single s.c. administration in both species. Danazol, because of its inherent insolubility, may serve as a delayed release system when administered by the s.c. or i.m. route and this novel formulation may extend the clinical utility of this compound.

Materials and methods

Rats

Sprague–Dawley female rats (200–250 g) were obtained from Taconic Farms Inc (Germantown, New York, USA), and were used for experimentation after establishment of normal cyclicity following daily vaginal lavage for 2 weeks. All animals were provided with food and water ad libitum and maintained in an environmentally-controlled room with an ambient temperature of 21°C and a 12 h light:12 h dark photo cycle. In addition to standard oral administration, experiments performed included either an s.c. injection, i.m. injection or a silastic capsule implant containing danazol. For all of the experiments, vaginal lavages were obtained daily on all rats to monitor their cycle and were continued until they returned to normal oestrous cyclicity. Slides were made of the lavage sample and the type of daily smear was recorded in the laboratory manual.

In a separate group of rats, both ovarian weight and histology as well as serum LH were investigated. Normally cycling rats were treated with either vehicle (n = 6) or the 400 mg/kg dose of s.c. danazol (n = 6). The animals were killed 10 days after treatment, serum was collected for LH determination and ovaries were weighed, fixed in Bouin’s solution and processed for haematoxylin and eosin

histology. All control animals were in dioestrus as were danazol-treated rats. LH was measured as described in our earlier study using reagents generously provided by the National Institute of Health (NIH) (Castracane et al., 1994). The average concentration of serum LH of four blood samples taken from each animal over a 1 hour period was determined in order to compensate for the pulsatile nature of LH secretion. The danazol used was synthesized at the Sterling Research Group (Rensselaer, New York, USA). For use in the injection experiment, danazol was diluted to 160 mg/ml in a vehicle of 10% ethyl alcohol and peanut oil and an emulsion was made using a mortar and pestle. A single depot injection of danazol of either 100 or 400 mg/kg body weight was given s.c. in a rear dorsal skin-fold. The same preparation at a dose of 400 mg/kg body weight was also administered i.m. in a hind limb muscle. All administrations of danazol vehicle were given on dioestrus and any effect on the rat oestrous cycle was determined by daily vaginal smears. In the silastic capsule implant study, dry danazol was packed in Dow Corning silastic brand medical grade tubing (602–285), the ends were then sealed with metal rivets and covered with Dow Corning silastic brand medical grade tubing (891, type A). The tubing used had an inner diameter of 1.58 mm and an outer diameter of 3.18 mm. The capsules were either 5 or 15 mm in length and were placed s.c. in the animal through a dorsal incision which was then sealed with a surgical staple. Control animals were implanted with empty capsules of similar size. The implantations were done under ether anaesthesia. Another group of animals was administered the same formulation of danazol at 100 or 400 mg/kg as a single gastric intubation. Control animals for this study included representatives of all of the treatment groups represented above, as well as untreated normally cycling rats for a total of 28 controls (see below).

**Monkeys**

Six normally cycling cynomolgus monkeys were used in this study. The animals had a history of regular menstrual cycles and a control menstrual cycle was followed prior to the administration of the single danazol s.c. injection. In our earlier study (Castracane et al., 1994) and in the rat study described above, we had used 400 mg/kg as a suspension in a single s.c. administration. Because of the much larger size of the cynomolgus monkey compared to a rat, the volume for injection was so large that we reduced the dosage to 200 mg/kg but at the same concentration as used in our earlier study. A single administration was performed near the end of the control menstrual cycle, from 4 days before to 5 days after the first day of menses. Animals were observed daily for signs of vaginal bleeding and blood samples were obtained at 2–3 day intervals during the baseline cycle before administration, during the interval following administration and for at least one cycle following menses. All blood samples were analysed for oestradiol and progesterone using commercially available methods (Diagnostic Products Corporation, Los Angeles, CA, USA). The intra- and interassay coefficients of variation for progesterone were 6.7 and <5% and for oestradiol were 7.9 and <5% respectively. Serum LH was measured by the mouse Leydig cell bioassay (Steiner and Bremner, 1981; Mann et al., 1987). Serum FSH was not measured because an appropriate monkey assay was not available at the time of this study. Changes in FSH in rhesus were expected to parallel those of LH. Results are presented for cycle length of the baseline control cycle, the interval of prolonged inhibition of menstrual cyclicity as indicated by the absence of menses, and for the first cycle after the prolonged suppression following danazol administration. Data are presented for individual monkeys to detail the endocrine changes during control cycles and in the post-treatment interval.

**Results**

**Rat oestrous cycle**

Normal animals in our colony generally have 4–5 day oestrous cycles. Controls in this study (n = 28) included a variety of combinations and were made up of animals receiving oral vehicle (n = 4), s.c. vehicle (n = 10), i.m. vehicle (n = 6), no treatment (n = 3), and blank silastic capsules (n = 5). In these controls, the mean ± SEM oestrous cycle of 4.7 ± 0.3 days was observed and in those animals who received oral danazol at 100 mg/kg, the mean interval to the next oestrous smear was 4.6 ± 0.4 days and with the higher dose (400 mg/kg), this interval was 4.3 ± 0.2 days. Danazol administered as a single s.c. injection of 100 mg/kg prolonged this oestrous interval to 10.0 ± 2.3 days (P <0.01) and at 400 mg/kg to 31.3 ± 2.0 days (P <0.001). Similar prolongation of this dioestrous smear was observed with i.m. administration of 400 mg/kg where this interval was extended to 27.7 ± 2.8 days (P <0.001). In those animals which received silastic capsules containing danazol, those with 5 mm capsules had an oestrous interval of 15.8 ± 3.9 days (P <0.001), and those with 15 mm silastic capsules had an interval of 12.5 ± 1.2 days (P <0.001). It was interesting that in both of these groups, the return of the oestrous smear was not always followed by a normal oestrous cycle but rather there seemed to be a prolongation of long cycles. In some animals, the prolongation of cycles went on for 30 days but with an occasional oestrous smear in between accounting for the shorter means. These effects on cycle length were summarized in Figure 1.

By 10 days after a single s.c. injection of 400 mg/kg danazol,
serum LH concentrations were reduced to 39.5 ± 4.6 ng/ml compared with 94.0 ± 12.0 ng/ml in control dioestrous animals (P < 0.01; data not shown). Furthermore, danazol administration resulted in a reduced mean ovarian weight of 67.0 ± 2.2 mg compared with controls, which were much heavier at 110.3 ± 4.9 mg (P < 0.001; data not shown). Histology of the ovaries showed fewer developing follicles and corpora lutea for animals treated with danazol compared with control ovaries (data not shown).

**Menstrual cycles in the cynomolgus monkey**

Mean (± SE) of menstrual cycle length for control cycles prior to danazol administration was 30.2 ± 1.4 days. After the single danazol administration, the next menses was prolonged to a mean duration of 117.5 ± 15.4 days and was significantly (P < 0.001) prolonged in comparison with the control cycle. In the first cycle which followed this prolonged inhibition, the mean cycle length was 33.7 ± 4.5 days which was not significantly different from the control cycle length and significantly shorter than the post-danazol interval (P < 0.001).

Individual and mean cycle length data are presented in Table 1. Endocrine data for the six individual monkeys are presented in Figures 2, 3 and 4.

**Monkey number 400**

There was a decline in serum bioactive LH that occurred after the administration of danazol. The first preovulatory oestradiol peak at about 125 days after the first blood sample occurred in the presence of low LH concentrations but was followed by a normal luteal phase. Subsequent cycles appeared to follow the normal pattern of oestradiol and progesterone. The interval from the control cycle menses to the post-treatment menses was 120 days with an apparently normal cycle of oestradiol and progesterone. An interference in the progesterone radioimmunoassay was seen within several days after the danazol administration and was evident in four of the six monkeys (numbers 400, 414, 419, 402).

**Monkey number 402**

There was a dramatic decline in bioactive serum LH following danazol administration with most concentrations at the limit of assay detection. Serum concentrations of oestradiol and progesterone declined within days of danazol administration and never exhibited normal menstrual cycle levels. The absence of menses persisted for 168 days following danazol administra-

**Table 1. Menstrual cycle length (days) in cynomolgus monkeys before and after a single s.c. danazol administration**

<table>
<thead>
<tr>
<th>Monkey ID no.</th>
<th>Baseline cycle length</th>
<th>Duration of post-danazol interval</th>
<th>Length of first cycle after menses</th>
</tr>
</thead>
<tbody>
<tr>
<td>400</td>
<td>29</td>
<td>120</td>
<td>33</td>
</tr>
<tr>
<td>402</td>
<td>26</td>
<td>168</td>
<td>32</td>
</tr>
<tr>
<td>414</td>
<td>33</td>
<td>108</td>
<td>35</td>
</tr>
<tr>
<td>419</td>
<td>35</td>
<td>72</td>
<td>54</td>
</tr>
<tr>
<td>423</td>
<td>28</td>
<td>84</td>
<td>23</td>
</tr>
<tr>
<td>452</td>
<td>30</td>
<td>153</td>
<td>25</td>
</tr>
<tr>
<td>Mean</td>
<td>30.2</td>
<td>117.5</td>
<td>33.7</td>
</tr>
<tr>
<td>SE</td>
<td>±1.4</td>
<td>±15.4</td>
<td>±4.5</td>
</tr>
</tbody>
</table>

A transient increase in serum LH was observed after danazol administration and soon declined but then increased prior to the next menses. A normal oestradiol and progesterone pattern was seen prior to the next menses and a cycle of normal endocrinology was seen after recovery. A menstrual hiatus of 108 days was observed in this monkey following danazol administration.

**Monkey number 414**

A transient increase in serum LH was observed after danazol administration and soon declined but then increased prior to the next menses. A normal oestradiol and progesterone pattern was seen prior to the next menses and a cycle of normal endocrinology was seen after recovery. A menstrual hiatus of 108 days was observed in this monkey following danazol administration.

**Monkey number 419**

A decline in serum LH following danazol administration was observed but soon an increase in LH was evident, resulting in the shortest interval for the animals observed, to the next menses of 72 days. An apparently normal pattern of oestradiol and progesterone followed danazol administration without menses and there was no normal pattern preceding the first post-treatment menses. The next intermenstrual interval was prolonged (54 days) and seemed to have an inappropriately long follicular phase before a normal oestradiol peak and luteal phase were observed.

**Discussion**

In an earlier study, we had demonstrated that a single s.c. injection of danazol (400 mg/kg) to the castrated male rat could effectively suppress the elevated serum concentrations of LH and FSH for >25 days (Castracane, et al., 1994). This interference with the negative feedback loop in the male was a most unexpected finding and suggests that danazol, probably because of its insolubility, is a natural sustained release preparation when injected by this route. Since danazol is used clinically for the treatment of endometriosis, we wished to investigate the effectiveness of this route of administration with normal ovarian cyclicity. We have performed two different studies, one to investigate the effectiveness of this route of administration of danazol on the cycling female rat and a second similar study to investigate this same approach in the normally cycling cynomolgus monkey. At the same time,
Figure 2. Serum concentrations of bioactive luteinizing hormone (LH) in the upper panels and serum oestradiol and progesterone in the lower panels. The two panels on the left present data for monkey no. 400 and on the right for monkey no. 402. In the upper panels, the arrows indicate menses (M) or the single danazol administration (D).

Figure 3. Serum levels of bioactive luteinizing hormone (LH) in the upper panels and serum oestradiol and progesterone in the lower panels. The two panels on the left present data for monkey no. 414 and on the right for monkey no. 419. In the upper panels, the arrows indicate menses (M) or the single danazol administration (D).
Prolonged effectiveness of s.c. danazol

Since there is such a pronounced prolongation of danazol action following the single s.c. injection, we thought it appropriate to investigate other routes which might also result in a prolonged duration of action. The first trial was to use the i.m. route since we would expect the same delayed release from the injection depot to the s.c. route. Using the 400 mg/kg of danazol, we found the mean duration to the next oestrous smear to be 27.7 ± 2.8 days and comparable with the same dose administered s.c. This suggests that the natural insolubility of danazol might be responsible for the prolonged release from either an s.c. or i.m. injection site.

Silastic capsules have been used for the sustained release of many steroids and are perhaps most recently of interest because of the development of Norplant, a silastic sustained-release preparation of levonorgestrol with a prolonged contraceptive effectiveness of up to 5 years (Sivin, 1988). We have prepared danazol silastic capsules of 5 and 15 mm, and monitored cycles in animals bearing these capsules. Danazol silastic capsule treatment resulted in an average return to an oestrous cycle after 15.1 or 12.3 days respectively, indicating enough danazol was released to have an effect on either the release of gonadotrophins or a direct ovarian action. More importantly in those animals with silastic capsules, when an oestrous cycle was observed, it was frequently followed by another prolonged anoestrous interval and that if a second capsule was implanted in some of these animals, longer cycles would follow the second implantation indicating that danazol can be released from a silastic capsule, but much more needs to be learned about the pharmacokinetics of release using this formulation.

In a recent report, Snyder et al. (1990) were able to show

because of the availability of the rat, we have chosen this species to investigate other routes of administration including the i.m. route and the use of silastic capsules, a common means for the tonic release of steroid hormones.

The normal oestrous cycle in the rat is generally 4–5 days in length and mean cycle lengths for controls were within the range expected (4.7 days). The oral administration of danazol in our earlier study had a transient inhibitory effect on serum gonadotrophins for 24–48 h with the doses employed. Using the same dosages from our earlier study, neither 100 nor 400 mg/kg when administered orally, had any effect on oestrous cycle length. Apparently the degree or duration of inhibition of LH and FSH was not sufficient to interfere with the oestrous cycle. These results are in good agreement with the relatively short period of inhibition of serum gonadotrophins in the castrated male when these same doses were used with this route of administration (Castracane et al., 1994).

When these same doses of danazol are administered by the s.c. route, the lower dose (100 mg/kg), was observed to extend the next oestrous cycle to >10 days and 400 mg/kg to >30 days. In our earlier study, the inhibition of LH in the castrated male with 100 mg/kg of danazol s.c. was only 24 h, but this route and dose results in a longer suppression of ovarian cycles (10.0 ± 2.3 days). In another study, adult ovariectomized rats injected s.c. with a total of 30 mg danazol had suppressed LH concentrations after 2 days of treatment (Kogo et al., 1992), but the prolonged effect of s.c. administration was not studied. Very few studies have used danazol administered by the s.c. route and these have generally been of inadequate dosage or duration to demonstrate the prolonged action of this depot danazol (Dmowski et al., 1971).

Figure 4. Serum levels of bioactive luteinizing hormone (LH) in the upper panels and serum oestradiol and progesterone in the lower panels. The two panels on the left present data for monkey no. 423 and on the right for monkey no. 452. In the upper panels, the arrows indicate menses (M) or the single danazol administration (D).
that danazol has both oestrogenic and androgenic properties which can be displayed under appropriate experimental protocols and that the androgenic aspects of danazol may interfere with vaginal cytology. Whether danazol interferes by its direct action on vaginal epithelium or directly through the effect on gonadotrophins or both, these results still represent a prolonged duration of action by this route of administration. In our earlier study (Castracane et al., 1994) where gonadotrophin suppression in castrated male rats was measured, the suppression of gonadotrophin activity lasted >25 days. In a small group of cycling females in this study, we have also demonstrated a suppression of serum LH 10 days after the initiation of treatment. There are numerous reports to suggest that danazol may have actions apart from the pituitary and suppression of gonadotrophin activity and the possibility for multiple sites of action in this prolongation of oestrous cyclicity cannot be discounted (Barbieri et al., 1979; Barbieri and Ryan, 1981). The observation that ovarian weight is reduced and, therefore, follicular development suppressed by treatment lends support to the possibility that gonadotrophin suppression may be the primary mechanism of action with these expected changes in the ovary.

In the present study we have also examined the s.c. route of administration for danazol in the cynomolgus monkey representing a more relevant non-human primate model for the human. Because of body size and shortage of supply of danazol, we used a smaller dose (200 mg/kg) than used in our rat studies. The results of this study in the monkey are in good agreement with our earlier rat studies. In Table I, the dramatic effect on menstrual cycle length is seen for the six monkeys in this study. Normal mean cycle length (30.2 days) with normal endocrine parameters was evident for all monkeys in the control interval prior to the administration of danazol. Following danazol administration, there was a clear decline in bioassayable LH in five out of the six monkeys. This is the expected biological action of danazol and indicates that danazol injected s.c. remains active over several months. Two monkeys exhibited apparently normal cycles in the interval immediately following danazol administration, but four out of the six monkeys exhibited an immediate cessation of the characteristic oestradiol and progesterone changes of the normal cycle. Prior to the next menses, which was delayed an average of 117.5 days, three monkeys demonstrated normal oestradiol and progesterone changes, indicating a normal cycle had resumed before the next menses, while three out of the six monkeys did not exhibit these expected endocrine changes. It could not be determined whether ovulation occurred. In five monkeys in whom blood sampling was continued after the reappearance of menses, four of the five exhibited predictable oestradiol and progesterone changes, indicative of a return to normal menstrual cyclicity, and a normal menstrual cycle length was observed in five of six of these monkeys, indicating that once the inhibitory actions of danazol have disappeared, subsequent cycles are normal.

It was observed that, immediately following danazol administration, there was a sharp but transient increase in immunoassayable progesterone which may be due to cross-reactions with danazol or danazol metabolites in the serum. The manufacturer of the progesterone kit indicated no cross-reaction with danazol; however, this represents the addition of danazol to the tube and it is well known that danazol produces a great variety of metabolites (Davison et al., 1976; Rosi et al., 1977) and that any or all of these metabolites may interfere with the radioimmunoassay. This short-lived increase in cross-reactants disappears in a few days and may represent a transient increase in serum concentrations following injection. Unfortunately, at the time of the study, there was no convenient method with which to monitor serum concentrations of danazol, or its metabolites, which would be most desirable in these monkeys.

There has been considerable conjecture concerning the role of danazol directly on the endometrium. Several studies have demonstrated binding of danazol to a variety of steroid receptors (Barbieri et al., 1979; Chamness et al., 1980) as well as a direct effect on endometrial cells in culture (Rose et al., 1988). This type of activity may account for the observed instances in several monkeys where apparently endocrinologically normal menstrual cycles were not associated with menses. No assessment of the degree of endometrial proliferation in cycles on danazol treatment was possible in these studies and would be an important aspect of any future studies with this route of administration.

Danazol studies in monkeys, as for humans and laboratory species, generally involves oral administration. In one study in which danazol was administered by i.m. injection to intact male Langur monkeys for 48 days, the effects on spermatogenesis were reversible only after 180 days (Gupta and Dixit, 1981). This may represent the prolonged duration of action of i.m. administered danazol which, based on the results of the present study, we would expect to show this effect. Conversely in castrated females, receiving 400 mg of danazol per day by gavage for 19 days, there was a rapid decrease in LH and FSH but a prompt return to pretreatment values 1–2 days after cessation of danazol treatment (Asch et al., 1979). This rapid return is the expected duration of effect of orally-administered danazol since no depot site remains as in a s.c. or i.m. administration.

The present studies agree with our earlier studies in castrated male rats, and demonstrate that danazol administered by injection, either i.m. or s.c., has an inordinately prolonged duration of action. The first report of this was our earlier study in castrated male rats where the duration of s.c. administered danazol on gonadotrophin concentrations persisted for >25 days (Castracane et al., 1994). We have now demonstrated a similar prolonged action in the inhibition of oestrous cycles in the rat which persisted for >30 days. We have also examined the s.c. route of administration of danazol in the cycling cynomolgus monkey and have demonstrated a prolonged inhibition of menstrual cycles in the cynomolgus monkey. The fact that a lower dose was effective in this species suggests that a dosage study is required to determine how low a dose of danazol will demonstrate this prolonged inhibitory action. These studies demonstrate that this route of administration has a previously undocumented prolonged duration of action, probably associated with the insolubility of danazol and the depot effect with either s.c. or i.m. administration. Two recent reports have also demonstrated interest in the use of other
routes of administration for danazol and include local injections of a danazol suspension into the uterine cervix (Takebayashi et al., 1995) and the vaginal administration of a danazol suppository (Mizutani et al., 1995). The use of a depot formulation of danazol might rejuvenate the use of a therapeutically beneficial compound resulting in low tonic levels, avoiding side-effects and decreasing the cost of administration. Lower serum concentrations may decrease side-effects and present the opportunity to decrease the cost of a month of danazol treatment with the ability to return this compound to the therapeutic arena from which it has recently been displaced.

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