Effects of Danazol on spermatogenesis in adult rats

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Summary. Adult male Wistar rats were treated with Danazol (4 mg/day s.c.) for 52 days. The drug produced a marked, rapid drop in serum testosterone concentrations to very low levels and caused a slower decrease in serum FSH, LH and testis weight. Flow cytometric analysis of testicular cell suspensions showed a decline in the absolute numbers of haploid cells (spermatids), tetraploid cells (mainly pachytene spermatocytes) and of cells in the S-phase of the division cycle, suggesting that Danazol inhibited proliferation of spermatogonia and/or primary spermatocytes. Histological counting of the different types of spermatogonia, however, revealed no significant change in their numbers during Danazol treatment. It is concluded that Danazol inhibited spermatogenesis primarily after the preleptotene stage of primary spermatocytes.

Introduction

Danazol (a 2,3-isoxazol derivative of 17α-ethinyl-testosterone) has been described as an active pituitary gonadotrophin inhibitory agent, suppressing the pituitary−gonadal axis in animals (Dmowski, Scholer, Mahesh & Greenblatt, 1971; Eldridge, Dmowski & Mahesh, 1974; Potts, Beyler & Schane, 1974; Pedroza, Vilchez-Martínez, Arimura & Schally, 1978; Dmowski, 1979; Dixit, Agrawal & Varma, 1981) and in man (Sherins, Gandy, Thorslund & Paulsen, 1971; Skoglund & Paulsen, 1973; Ulstein, Netto, Leonard & Paulsen, 1975; Paulsen & Leonard, 1976). Danazol appeared to suppress spermatogenesis by depletion of spermatocytes, spermatids and spermatozoa (Sherins et al., 1971), but the stage or stages of spermatogenesis which are affected have not yet been defined. Ulstein et al. (1975) suggested that Danazol acts by inhibiting spermatogenesis at the spermatocyte/spermatid level. In most studies (Dmowski et al., 1971; Eldridge et al., 1974; Pedroza et al., 1978), animals without functional gonads or immature rats were used to assess the antagonadotropic properties of Danazol. According to Barbieri et al. (1977), these studies are not directly applicable to animals with intact gonadal function. At all tested doses (1.25–10 mg/kg s.c.) of Danazol in male rats, serum LH and FSH concentrations were unsuppressed, whereas the suppression of serum testosterone appeared to be dose-dependent (Barbieri et al., 1977).

The purposes of the present investigation were (1) to study the antagonadotropic effects of Danazol in intact mature male rats by measuring serum gonadotrophin concentrations, and (2) to examine the effect on the different steps of spermatogenesis by counting the different types of spermatogonia. In earlier published reports (Dmowski et al., 1971; Eldridge et al., 1974; Pedroza et al., 1978) Danazol had antagonadotropic effects in rats at a dosage of 4 mg s.c. given daily, and so this dose was used throughout the present study.

Materials and Methods

The experiments were started with 3-month-old male outbred Wistar rats (Cpb:WU) with a body

weight of about 300 g. The animals were allowed free access to dry pellet food (RMH-TM HOPE-Farms, Woerden, The Netherlands) and tap water. They were housed in groups of 4 in well ventilated cages at 22°C with a day/night rhythm of 12 h. The 42 rats received daily (at 09:00 h) s.c. injections of 4 mg Danazol (Sterling-Winthrop Research Institute, London, U.K.) suspended in 0·2 ml sesame oil. Groups of 6 rats were killed at 10:00 h under ether anaesthesia before the injection (controls) and at 7, 14, 21, 28, 39 and 52 days after the start of drug injections. Blood was collected by heart puncture and the testes were removed immediately and weighed. After centrifugation (1200 g, 10 min), serum samples were stored at −20°C until analysed.

**Hormone measurements.** Serum concentrations of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were measured by double-antibody solid-phase radioimmunoassays using the rat gonadotrophin kit reagents kindly supplied by NIAMDD, Bethesda, Maryland, U.S.A. The measurements were expressed as ng/ml in terms of the NIAMDD-rat FSH-RP-1 and LH-RP-1 standards, respectively. The minimum detectable concentrations were 10 ng LH/ml and 50 ng FSH/ml. The precision was 5·9% within assays and 6·7% between assays for duplicate measurements of FSH in a serum pool (mean: 264 ng/ml in 15 consecutive assays). For LH these values were, respectively, 3·9% and 9·6% (mean: 59 ng/ml in 15 consecutive assays).

Testosterone was measured by a dextran-coated charcoal radioimmunoassay after extraction of serum samples with diethyl ether. The antiseraum used was raised in a rabbit and directed against testosterone-3-(O-carboxymethyl)-oxime–BSA. The sensitivity of the assay was 0·1 µmol/ml and the precision, as calculated from a serum pool (mean value: 1·85 µmol/ml after 14 consecutive measurements), was 3·9% within assays and 5·7% between assays.

**Histology.** The left testis from each animal was fixed in Bouin’s fluid, dehydrated in 70% ethanol and embedded in paraffin wax. Duplicate transverse sections of 5 µm were taken from the midportion of the testis and stained with periodic acid–Schiff (PAS) with and without prior diastase digestion. For histological measurement, the germ cell maturation sequence of 14 stages as proposed by Leblond & Clermont (1952) was used. The counts were made on cross-sections of 25 seminiferous tubules. The numbers of spermatogonia or preleptotene spermatocytes were expressed as numbers per 100 Sertoli cells. Expressing the data as numbers of cells per 100 Sertoli cells corrected for tissue shrinkage and permitted a comparison of germ cell counts from one time interval to another. Spermatogonia of types A0 and A1 were scored as a single class of cells since in our material they could not be differentiated. Type B spermatogonia were scored at stage 6; intermediate spermatogonia at stage 3 and preleptotene spermatocytes at stage 8 of the cycle.

**Flow-cytometry.** The right testis from each animal was dissected free from fat and connective tissue. After decapsulation, the tissue was minced carefully with scissors in 15 ml phosphate buffer (pH 7·4; 23·8 mg PO4³⁻/ml) and sieved through a 50 µm mesh filter. All preparation steps were done at room temperature. This cell suspension was passed through a 25-gauge, 25 mm needle, centrifuged at 370 g for 10 min and the cell pellet resuspended in 10 ml phosphate buffer by vortexing. Debris was removed by filtration through a nylon filter (pore size: 50 µm). The suspension was again centrifuged at 370 g for 10 min and the supernatant discarded. Samples were fixed in 50% ethanol (−20°C) and stored at 4°C until analysed. Staining was carried out as described elsewhere (Tannenbaum, Cassidy, Alabaster & Herman, 1978; Hamilton, Habbasser & Herman, 1980). Briefly, 1 ml cell suspension (about 3 × 10⁶ cells) was centrifuged for 10 min at 370 g and the ethanol was discarded. The cell pellet was resuspended in 1 ml staining solution consisting of 0·1 mg Mithramycin (Serva Fein Biochemica, Heidelberg, West Germany) per ml 15 mM-MgCl₂ in 0·85% NaCl (w/v). Staining was carried out in the dark at room temperature for 30 min. Flow-cytometric analysis was performed on an Ortho System 50-H (Ortho Instruments; Westwood, MA, U.S.A.) using the 457 nm line of the argon ion laser at 200 mW power. A Coulter counter was used to count the number of cells in 1 ml of the cell suspension. The total cell counts per volume were needed for calculation of the total number of the different cell types in the testis.
Statistics. Statistical analysis was performed by using the two-sided Mann–Whitney U test. Differences were considered to be statistically significant when $P < 0.05$.

Results

Serum hormone concentrations

There was a significant decrease of serum FSH, LH and testosterone concentration after 7 days.

![Graphs showing changes in serum hormone concentrations](image)

**Fig. 1.** The effects of Danazol (4 mg/day s.c.) on serum concentrations of (a) FSH, (b) LH and (c) testosterone in adult male rats. Values are mean ± s.d. for 6 rats/group.

![Graph showing changes in testicular weight](image)

**Fig. 2.** The effect of Danazol (4 mg/day s.c.) on mean ± s.d. testicular weights of adult rats (6/group).
Fig. 3. The numbers (mean ± s.d.) of the different types of spermatogonia, A (d), intermediate (c), and B (b), and preleptotene primary spermatocytes (a) per 100 Sertoli cells.

Fig. 4. DNA fluorescence distribution from a single cell suspension of a rat testis. The first two peaks, (1a,1b) represent haploid cells (round and elongated spermatids respectively), the third (2c) and fourth (4c) peaks represent diploid and tetraploid cells, respectively. Mithramycin stain, variation coefficient (first peak) = 3.5%.

of Danazol treatment ($P < 0.05$), which persisted throughout the period of Danazol administration (Fig. 1). Also a significant ($P < 0.05$) decrease in total testicular weight was observed (Fig. 2) after 39 days of drug administration.

**Germ cell populations**

No significant effects of Danazol on the numbers of the different types of spermatogonia or on the numbers of preleptotene primary spermatocytes were detected (Fig. 3).

**Proliferative activity of testicular cells**

An example of a flow-cytometric histogram of untreated testicular cells is presented in Fig. 4.
The two subpeaks in the 1C region of the histogram represented round (1a) and elongated (1b) spermatids respectively. The geometry of the histograms was symmetrical and distributions were obtained with a coefficient of variation of 3.5% (1C peak). A decrease of total number of haploid cells was observed ($P<0.05$) after 28 days of treatment (Fig. 5) and an almost parallel decline of tetraploid cells occurred (Fig. 5). A significant decrease ($P<0.05$) of the number of cells in the S-phase (Fig. 5) was seen after 39 days of Danazol administration.

**Discussion**

It is apparent from the present study that Danazol effectively suppresses the pituitary–gonadal axis in adult rats. In our study a significant decrease of gonadotrophins occurred ($P<0.05$) within 7 days of Danazol treatment at a dose of 4 mg/day s.c. From these data it appears that Danazol treatment of animals with intact gonadal function is accompanied by lowered hormone concentrations (Barbieri et al., 1977).

Histological counting showed no significant influence of Danazol treatment on the different types of spermatogonia. This supports the evidence that anti-gonadotrophic treatments have similar effects on the specific stages of spermatogenesis (Russell, Malone & Karpas, 1981). Of special interest, however, is the quantitative analysis of changes in proliferative activity of testicular cells in animals receiving Danazol. In normal spermatogenesis the last duplication of DNA takes place in the S-phase of a primary spermatocyte (Monesi, 1962). The number of cells in the S-phase therefore reflects the proliferative activity of cells in the early stages of spermatogenesis. From the decrease of cells in the S-phase during Danazol administration (Fig. 5), it can be concluded that the drug influenced the proliferative activity of spermatogonia and/or primary spermatocytes, either directly or indirectly. Whether this was due to a decrease in spermatogonia or in preleptotene spermatocytes, or both remains to be investigated. Tetraploid cells consisted mainly of pachytenic spermatocytes and only to a lesser degree of spermatogonia in mitosis (van Kroonenburgh, van Gasteren, Beck & Herman, 1986). Finally, during Danazol treatment a drop in total numbers of cells takes place (Fig. 5). The uncoupling of the effects on the total number of cells in the S-phase
from effects on gonadotrophin concentrations suggests that, at least initially, the early stages of spermatogenesis are relatively independent of serum concentrations of FSH, LH and testosterone.

It can be concluded that Danazol suppresses serum concentrations of gonadotrophins in intact adult male rats and inhibits spermatogenesis from the preleptotene stage of the primary spermatocyte onwards. It also has a direct or indirect inhibiting effect on the proliferation of spermatogonia and/or primary spermatocytes.

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References


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