ACTION OF AMPHOTERICIN B (FUNGIZONE) ON SPERMATOGENESIS IN THE RABBIT

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Summary. Quantitative and qualitative analysis of the seminiferous epithelium of rabbits treated with Amphotericin B (Fungizone) revealed that ten intravenous injections of Fungizone, at a dose level of 2.0 mg Fungizone/kg body weight/day, had no effect on the duration of the stages of the cycle of the seminiferous epithelium, but affected the spermatozoa prior to their release from the seminiferous tubules. Fungizone decreased the rate at which the spermatozoa migrated from the Sertoli cells towards the lumen. Thus, spermatozoa which normally are released prior to Stage 1 were retained in some Stage 1 tubules of injected animals. A single local injection of Fungizone, at a dose level of 0.2 mg/kg, into the cava vaginalis did not result in any observable change of the seminiferous epithelium. Unilateral castration had no effect on spermatogenesis in the remaining testis.

INTRODUCTION

During the last decade investigations have revealed that certain chemical agents can interfere with the process of spermatozoan formation. Certain antifertility agents destroy the germinal epithelium completely, while others produce reversible changes. In rodents, subcutaneous administration of cadmium salts produced an irreversible destruction of the germinal epithelium and partially reversible destruction of the interstitial cells (Pařízek & Záhoř, 1956; Pařízek, 1957, 1960). In rats and rabbits, triethylenemelamine, Busulphan, and other alkane sulphonic esters have been shown to selectively interfere with spermatogenic cells (Jackson, Craig & Fox, 1959; Jackson, Fox & Craig, 1962; Steinberger, 1962; Fox, Jackson, Craig & Glover, 1963). Oral administration of highly chlorinated naphthalene to bulls has been reported to lead to reversible interference with spermatogenesis (Olson & Skidmore, 1954; Vlahos, McEntee, Olañson & Hansel, 1955).

In-vitro studies by R. H. Foote (1961—unpublished data) revealed that the antifungal antibiotic, Amphotericin B (Fungizone), was highly spermicidal. Less than one part per million was lethal to bovine spermatozoa within 24 hr. This observation led to studies of the effects of Amphotericin B on spermatogenesis in the rabbit.

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MATERIAL AND METHODS

Three experiments were carried out to determine the effect of intravenous and local (intra-cava vaginalis) injections of Amphotericin B (Fungizone, Squibb) on spermatogenesis in the rabbit.

EXPERIMENT 1

Intravenous injections in New Zealand rabbits

Eight New Zealand rabbits averaging 4200 g (range 3540 to 4970 g) in body weight were paired on the basis of body weight. One member of each pair was assigned at random to a control (A-1) or a treated (A-2) group. Group A-2 animals received ten injections of Fungizone over an 11-day period at the daily rate of 2.0 mg/kg of body weight. The first five injections were given in the marginal vein of the ear. Due to thrombophlebitis and other complications with this vein the sixth injection was given intraperitoneally and the four remaining ones in the cephalic vein. One testis, randomly selected, was removed from each animal at the beginning of the experiment and 2 days after the last injection the animals were killed. Thus, the second testis was removed 13 days after the first one. The tissues from one animal were lost in processing, leaving three animals in this group.

EXPERIMENT 2

Intravenous injections in Dutch-Belted rabbits

Nine Dutch-Belted rabbits averaging 2140 g (range 1890 to 2410 g) in body weight were used in this experiment. Four animals served as controls (Group B-1) and the remaining five (Group B-2) were scheduled to receive ten intravenous injections of Fungizone in the marginal ear vein over an 11-day period at the daily rate of 2 mg/kg per injection. Each Fungizone injection was followed by an injection of 1 ml physiological saline solution in order to wash the Fungizone from the vein. This procedure reduced the incidence of thrombophlebitis considerably. One treated animal died within a few minutes after receiving the sixth injection of Fungizone, and was omitted from the experiment. As in Experiment 1 both groups were unilaterally castrated at the beginning of the experiment and killed 2 days after the last injection.

EXPERIMENT 3

Local injection in Dutch-Belted rabbits

Twelve Dutch-Belted bucks, averaging 2130 g (range 1860 to 2265 g) in body weight, were randomly divided into three groups of four (C-1, C-2 and C-3). One randomly selected testis of each animal served as control and an injection was made into the cava vaginalis of the other side by carefully inserting a 24-gauge needle between the two tunics. Group C-1 served as a sham-operated control group and was injected with 0.04 ml/kg of 5% dextrose solution. All animals in Groups C-2 and C-3 received 0.2 mg/kg of Fungizone, contained in 0.04 ml of a 5% dextrose solution. The animals in Groups C-1 and C-2 were killed 13 days, and those in Group C-3 25 days, after the injections were given.
Action of Fungizone on spermatogenesis in rabbit

GENERAL PROCEDURE

Unilateral castrations and injections into the cava vaginalis were carried out while the animals were under pentobarbital and/or ether anaesthesia. In addition all Dutch-Belted rabbits were injected with 1 ml of procaine (2% procaine and 1:100,000 dilution of epinephrine) at the base of the scrotum on the side from which the testis was removed, or locally injected. Prior to surgery the scrotal area was disinfected with Zepharin (1:1000 dilution).

The castrations were performed by making a 2-cm long skin incision at the base of the scrotum and exposing the spermatid cord. The cord was clamped with two haemostats, a ligature was placed proximal to them, and the spermatic cord was severed distal to the ligature by rotating one haemostat. A single silk suture was placed in the scrotum. Each animal received an intramuscular injection of ‘Combiotic’ (Chas. Pfizer & Co) containing 100,000 units of penicillin and 0.125 g of dihydrostreptomycin.

The Fungizone for intravenous injections was prepared by adding 25 ml of sterile 5% dextrose solution to the vials containing 50 mg of Fungizone and a sodium desoxycholate solubilizer. Solutions were prepared daily before injection. Each animal received 1 ml of solution (2-0 mg of Fungizone)/kg body weight/day.

At the time the testes were removed from the animals, semen smears were prepared from the tail of each epididymis. These smears were evaluated on the basis of motility of the spermatozoa. Testis weight was recorded after removing the epididymis.

In the case of the New Zealand rabbits (Groups A-1 and A-2) tissue samples were taken from two randomly selected areas of the testes. In all other cases (Groups B-1, B-2, C-1, C-2 and C-3) tissue samples were taken from two fixed locations near the opposite poles of each testis. After fixation and embedding 8-μ-thick histological sections were prepared. The periodic acid–Schiff (PAS)–haematoxylin technique was employed for staining the sections.

A quantitative method of analysis of the germinal epithelium was used in evaluating the treatment effects. This was based on a division of the cycle of the seminiferous epithelium into eight stages described previously (Swierstra & Foote, 1963).

The number of seminiferous tubules classified per location was sixty for Groups A-1 and A-2, and eighty for all other groups. Only tubules cut at right angles to the axis of the seminiferous tubule were classified.

RESULTS AND DISCUSSION

Unilateral castration had no effect on testis weight. The testes removed from New Zealand rabbits on Day 0 averaged 2.92 g, and the remaining testes removed on Day 13 averaged 2.93 g. Corresponding values for the Dutch-Belted rabbits were 1.93 and 2.02 g.

Also, unilateral castration had no effect on the frequency of the stages of the seminiferous epithelium (A-1, B-1, Table 1) of the remaining testis measured 13 days after the first testis was removed (P>0.25), or on the composition of the cell populations of the eight stages. From these observations it was concluded that
tests removed from animals by unilateral castration prior to injection of Fungizone were valid controls for measuring treatment effects in the same animals.

Intravenous injections of Fungizone resulted in a change in frequency of a number of stages of the seminiferous epithelium (A-2, B-2, Table 1). In Group A-2 the frequency of Stage 7 increased from 16.9 to 43.1%, while the frequency

of Stage 1 decreased from 26.9 to 6.9. In Group B-2 the frequency of Stage 7 increased from 17.3 to 50.6% and the frequencies of Stages 8, 1 and 2 decreased (12.8 versus 4.1, 27.8 versus 1.1 and 10.2 versus 5.2, respectively). These changes in both groups represent significant departures from the normal (P<0.01).

To explain the increase in Stage 7 and the decreases in Stages 8 and 1 (also Stage 2 in Group B-2), it is helpful to reconsider the criteria used for defining these stages (Swierstra & Foote, 1963).

Stage 7: Extends from the beginning to the end of the movement of the spermatozoa towards the lumen.

Stage 8: Extends from the time the spermatozoa line the lumen to their complete disappearance from the lumen.

Stage 1: Extends from the absence of spermatozoa in the lumen to the beginning of the elongation of the spermatids.

Stage 2: Extends from the beginning of elongation to the end of elongation of the spermatids.

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**Table 1**

<table>
<thead>
<tr>
<th>Frequency of the stages (%)</th>
<th>New Zealand rabbits</th>
<th>Dutch-Belted rabbits</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stage</strong></td>
<td>Control (A-1)</td>
<td>Fungizone injected (A-2)</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>---------------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td></td>
<td>Testes removed Day 0</td>
<td>Testes removed Day 0</td>
</tr>
<tr>
<td>1</td>
<td>24.8</td>
<td>24.4</td>
</tr>
<tr>
<td>2</td>
<td>10.2</td>
<td>11.6</td>
</tr>
<tr>
<td>3</td>
<td>4.4</td>
<td>3.7</td>
</tr>
<tr>
<td>4</td>
<td>10.6</td>
<td>12.5</td>
</tr>
<tr>
<td>5</td>
<td>5.2</td>
<td>3.8</td>
</tr>
<tr>
<td>6</td>
<td>13.4</td>
<td>14.4</td>
</tr>
<tr>
<td>7</td>
<td>17.3</td>
<td>17.9</td>
</tr>
<tr>
<td>8</td>
<td>14.2</td>
<td>11.7</td>
</tr>
</tbody>
</table>

Tubules classified per testis
- 120
- 120
- 120
- 120
- 160
- 160
- 160
- 160

Total tubules classified
- 480
- 480
- 360
- 360
- 640
- 640
- 640
- 640

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A careful analysis of the seminiferous tubules in these four stages revealed that intravenous injections of Fungizone selectively interfered with the movement of spermatozoa from the Sertoli cells to the lumen of the seminiferous tubules, and also with the release of spermatozoa from the lumen of the tubule. Since the migration of spermatozoa from the Sertoli cells to the lumen of the tubule was used as a criterion to delimit Stage 7, such interference would be expected to increase the frequency of this stage, and decrease the frequency of the stages immediately following it. It was observed that the population of cells other than spermatozoa in some of the tubules classified as Stage 7 were characteristic of tubules in Stages 8, 1 and 2 in normal animals.

On the basis of these observations it was decided to re-define Stages 7, 8 and 1, using as criteria spermatogenic cells other than spermatozoa. The new criteria were selected so as to give clearly definable stages, while keeping the duration of each stage similar to its duration under the original system of classification. This slightly modified scheme made it possible to evaluate on a quantitative basis the effect of Fungizone on the synchronization of the spermatogenic process and to observe what effect the chemical had on the composition of the cell populations making up each of the eight stages. The revised scheme of classification was as follows:

**Stage 7**: Extends from the appearance of Type B spermatogonia to the appearance of preleptotene primary spermatocytes. During this interval the spermatids reveal a distinct acrosome covering approximately one-third of the anterior portion of the nucleus in preparations stained with PAS and haematoxylin.

**Stage 8**: Extends from the appearance of preleptotene primary spermatocytes to the appearance of the leptotene primary spermatocytes.

**Stage 1**: Extends from the appearance of the leptotene primary spermatocytes to the time the spermatids start to elongate.

The results obtained using this modified scheme of classification for the treated testes are presented in Table 2. A chi-square analysis of the combined data from Groups A-2 and B-2 showed that there was no significant difference ($P > 0.25$) between the control and the treated testes with respect to the frequency of the eight stages of the cycle of the seminiferous epithelium. From this it was concluded that Fungizone did not affect the synchronized initiation of successive cycles, but delayed release of the spermatozoa.

The deviations of the cellular pattern of Stages 8 and 1 from normal are illustrated by the photomicrographs on Plate 1; Fig. 1 is a Stage 8 control testis and Fig. 2 a Stage 8 testis after Fungizone administration. With the exception of the location of the spermatozoa both tubules have a similar cellular pattern. Sertoli cells, Type A spermatogonia and preleptotene primary spermatocytes line the basement membrane. Pachytenic primary spermatocytes and spermatids are similarly arranged in both tubules. In Pl. 1, Fig. 1 there still are a few Type B spermatogonia present indicating that this particular tubule is not as far advanced along the cycle of the seminiferous epithelium as the one in Pl. 1, Fig. 2, even though the spermatozoa have migrated to the lumen of the tubule in Pl. 1, Fig. 1.

Pl. 1, Figs. 3 and 4 show Stage 1 testes removed from control and treated animals, respectively. Sertoli cells, Type A spermatogonia and leptotene primary...
Table 2
DURATION (FREQUENCY) OF THE STAGES OF THE CYCLE OF THE SEMINIFEROUS EPITHELIUM OF NEW ZEALAND AND DUTCH-BELTED RABBITS IN EXPERIMENTS 1 AND 2 (REVISED METHOD OF CLASSIFICATION)

<table>
<thead>
<tr>
<th>Stage</th>
<th>New Zealand rabbits injected with Fungizone (A-2)</th>
<th>Dutch-Belted rabbits injected with Fungizone (B-2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Testes removed on Day 0</td>
<td>Testes removed on Day 13*</td>
</tr>
<tr>
<td>1</td>
<td>26-9</td>
<td>27-2</td>
</tr>
<tr>
<td>2</td>
<td>9-2</td>
<td>12-8</td>
</tr>
<tr>
<td>3</td>
<td>4-4</td>
<td>6-4</td>
</tr>
<tr>
<td>4</td>
<td>12-2</td>
<td>9-7</td>
</tr>
<tr>
<td>5</td>
<td>5-6</td>
<td>5-0</td>
</tr>
<tr>
<td>6</td>
<td>13-9</td>
<td>12-2</td>
</tr>
<tr>
<td>7</td>
<td>16-9</td>
<td>12-8</td>
</tr>
<tr>
<td>8</td>
<td>10-8</td>
<td>13-9</td>
</tr>
</tbody>
</table>

| Tubules classified per testis | 120 | 120 | 160 | 160 |
| Total tubules classified      | 360 | 360 | 640 | 640 |

* Revised method of classification used for testes removed after treatment on Day 13.

Table 3
DURATION (FREQUENCY) OF THE STAGES OF THE CYCLE OF THE SEMINIFEROUS EPITHELIUM OF DUTCH-BELTED RABBITS IN EXPERIMENT 3

<table>
<thead>
<tr>
<th>Stage</th>
<th>Frequency of the stages (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dextrose group†</td>
</tr>
<tr>
<td></td>
<td>Control testes</td>
</tr>
<tr>
<td>1</td>
<td>25-3</td>
</tr>
<tr>
<td>2</td>
<td>9-4</td>
</tr>
<tr>
<td>3</td>
<td>5-5</td>
</tr>
<tr>
<td>4</td>
<td>14-4</td>
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<tr>
<td>5</td>
<td>5-0</td>
</tr>
<tr>
<td>6</td>
<td>12-3</td>
</tr>
<tr>
<td>7</td>
<td>16-4</td>
</tr>
<tr>
<td>8</td>
<td>11-7</td>
</tr>
</tbody>
</table>

| Tubules classified per testis | 160 | 160 | 160 | 160 |
| Total tubules classified      | 640 | 640 | 640 | 640 |

* Original method of classification used.
† Control testes removed on Day 0, injected testes on Day 13.
‡ Control testes removed on Day 0, injected testes on Day 25.
Cross-sections of seminiferous tubules of control and Fungizone-treated rabbits. All Figs. × 550.

A = Type A spermatogonium, B = Type B spermatogonium, M = basement membrane,
P = preleptotene primary spermatocyte, Y = leptotene (young) primary spermatocyte,
O = pachytene (old) primary spermatocyte, T = spermatid, Z = spermatozoon,
S = Sertoli cell.

Fig. 1. Stage 8 of the control testes.
Fig. 2. Stage 8 of the testes after intravenous administration of Fungizone.
Fig. 3. Stage 1 of the control testes.
Fig. 4. Stage 1 of the testes after intravenous administration of Fungizone.

(Facing p. 18)
spermatocytes line the basement membrane in both tubules. Also, both tubules have similar arrangements of the pachytene primary spermatocytes and spermatids. However, in the control tubule all spermatozoa have left the lumen, while in the Fungizone-treated tubule the spermatozoa are still present among the spermatids.

It has yet to be established whether or not Fungizone affected the rate of migration of spermatozoa from the Sertoli cells to the lumen of the tubule by killing the spermatozoa. Many of the spermatozoa present in Stages 1 and 2 were morphologically abnormal. Some appeared to be degenerating, indicating that Fungizone kills the spermatozoa in vivo, as it does in vitro. Experiments with longer periods after injection are needed to evaluate semen and spermatozoa during their passage through the epididymis. In the present study affected spermatozoa did not have sufficient time to reach the tail of the epididymis, where sperm motility was the same in control and Fungizone-treated animals.

The effect of injecting Fungizone into the cava vaginalis is shown in Table 3. The dextrose-injected group served to measure any possible effects of the injection procedure. Statistical analyses revealed no significant differences ($P > 0.1$) between the control and injected testes in any group. The lack of any Fungizone effect in this experiment was probably due to a failure of the Fungizone to be absorbed from the cava vaginalis in sufficient amounts to provide an effective dose. Deposits with the characteristic yellow colour of the injected Fungizone were present in the cava vaginalis at the time the animals were killed. The surgical procedures did not alter spermatogenesis.

ACKNOWLEDGMENT

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REFERENCES


