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Hormonal Regulation of Spermatogenesis in the Hypophysectomized Rat: Quantitation of Germ-Cell Population and Effect of Elimination of Residual Testosterone After Long-Term Hypophysectomy

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ABSTRACT: Spermatogenesis continues after long-term hypophysectomy (Hx), but massive cell degeneration prevents seminiferous tubules from attaining the full complement of cells. One objective of this study was to determine the vulnerable sites for completion of spermatogenesis in long-term Hx rats. It is now known that Leydig cells continue to secrete small amounts of androgen after Hx. A second objective was to determine the cellular sites that are maintained by residual androgen secreted by Leydig cells post-Hx. Two groups of adult animals were utilized. Both groups were Hx for 36 days, but one group of rats received the androgen antagonist flutamide during the 26th through the 36th day of Hx (10 days). Germ-cell numbers were quantified using a method that allowed their expression as numbers of cells present per hour of development. In the long-term Hx rat, the germ-cell population increased to preleptotene, but the divisions that led to preleptotene were inefficient due to cell degeneration. Subsequent to preleptotene, there was a gradual loss in cells such that there were few germ cells remaining by steps 9–13. Flutamide given to Hx rats did not result in a significant difference in the numbers of intermediate and type B spermatogonia or significant differences in progenitor cells. A significant and major depression of cell numbers in Hx-flutamide–treated rats occurred in the cell division of type B spermatogonia to form preleptotene spermatocytes. There was a less dramatic, although significant, depression of cell numbers in Hx-flutamide–treated animals that occurred from preleptotene until late pachytene as well as an increased loss of round spermatids at midcycle (step 5–6). These data demonstrate that cell loss after long-term Hx occurs at numerous phases of spermatogenesis. The data also demonstrate that the presence of residual androgen action after long-term Hx results in enhanced germ-cell survival. Although the major blockage in cell viability occurs at midcycle steps in the long-term Hx rat, there are several other hormone-sensitive phases of spermatogenesis.

Key words: Testis, germ cells, androgen, morphometry.
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Hypophysectomy (Hx) has been long known to cause testicular regression (Smith, 1927; Clermont and Morgentaler, 1955) due to cessation of secretion of luteinizing hormone (LH), follicle-stimulating hormone, and possibly other hormones (reviewed by Sharpe, 1994). Regression of the rat testis after long-term Hx has been described (Clermont and Morgentaler, 1955). In the cited study, spermatogenesis did not proceed beyond the round spermatid phase of development in the absence of the pituitary in the adult rat Hx for 36 and 61 days. Cells less mature than round spermatids were reduced in number as well, indicating that there were blockages to spermatogenesis other than in the transition from a round spermatid to an elongate spermatid. There has never been a thorough study to determine the specific vulnerable sites that result in quantitative deficiency in spermatogenesis in the long-term Hx rat, although such studies exist for short-term (Russell and Clermont, 1977) and intermediate-term Hx (El Shennawy et al, 1998; this volume).

It was recently shown that Hx does not cause the complete cessation of testosterone (T) production and secretion (reviewed by Sharpe, 1994). This phenomenon appears to be due to the ability of Leydig cells to secrete small amounts of T in the absence of LH stimulation. Thus, what had been thought to be complete regression of the testis by numerous investigators who utilized Hx prior to the mid-1990s might have been a condition in which the animal's Leydig cells secreted low levels of T that acted to support spermatogenesis. It has been shown that flutamide, an androgen-receptor competitor, can cause regression of the testis beyond what has been attributed to Hx alone (reviewed by Sharpe, 1994) or, for that matter, to other conditions that affect LH (Chandolia et al, 1991).

The long-term Hx rat, thus, can serve as a model to

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study androgen action in the testis. This study employed flutamide, an agent that competitively blocks the androgen receptor (Peets et al, 1974) and that will enhance post-Hx regression of the testis (reviewed by Sharpe, 1994). Although flutamide blocks androgen action, this study examined the relative loss of androgen in an already androgen-deprived system (Hx). The present study employed a novel method for quantification of cell populations in the testis. Compared with Hx alone, the flutamide-treated animal contained fewer germ cells and showed a major block to spermatogenesis at midcycle. However, the blockage to spermatogenesis was not only at midcycle since several other vulnerable sites were noted.

Methods

Animals

Male Sprague-Dawley rats from Harlan Labs (Indianapolis, Indiana) were used for the study. Hypophysectomies were performed by the supplier via the transauricular route with a stereotaxic apparatus on adult rats weighing 300 g. Animals were provided with water containing 5% sucrose and solid food pellets *ad libitum* after surgery. Animals were shipped to Southern Illinois University after a 3-day period and were housed in the vivarium where lighting was programmed at 14 hours of light to 10 hours of dark, and 5% sucrose water was supplied.

Experimental Design

Seven Hx rats were randomly selected for each group; however, questions about the adequacy of fixation of the testis left only six animals in the Hx-flutamide–treated group. At 26 days post-Hx, the general time post-Hx that maximal testis regression occurs (Clermont and Morgentaler, 1955), one group of animals was administered flutamide (Eulexin; Schering Corporation, Kenilworth, New Jersey). Flutamide was administered twice daily using 2.5 mg, for a total of 5 mg/day, as a suspension in 0.9% sodium chloride and 1% gelatin. Preliminary studies in our laboratory indicated that 2.5 mg administered twice daily was the lowest dosage that maximally reduced testis weight in a long-term Hx rat. It has been shown that after about 25 days of Hx, the numbers of germ cells in the testis stabilize, and no further loss occurs (Clermont and Morgentaler, 1955). Administration of flutamide continued for 10 days after the period that testis weights had stabilized. All animals were sacrificed on the 36th day after Hx.

Tissue Preparation and Tubular Diameter Measurements

Tissue was prepared according to the methods of El Shennawy et al (1998; this volume). Tubular diameters were measured using an ocular micrometer calibrated with a stage micrometer. Profiles of 20 round or near-round tubules were measured at ×150 magnification at random for each animal, and mean values were calculated. Only the short axis of each tubule was measured.

Quantitation of Viable Germ Cells

A method devised for determining the numbers of viable germ cells per unit period of time was utilized (see El Shennawy et al, 1998; this volume). Counts of viable cell types at the various stages of spermatogenesis employed morphometric techniques to determine the numbers and kinds of germ cells at key stage groupings of the rat spermatogenic cycle. Tissue sections were examined under ×500 magnification on a binocular Nikon Microphot-FX (Nikon, Garden City, New York) microscope. The classification of spermatogenesis used to describe stages of the cycle was that of Russell et al (1990). The four stage groupings and the identifying cell types utilizing the point counting method were as follows. Stage group II–IV was identified by the presence of intermediate spermatogonia and the accompanying step 2 and step 4 spermatids. Tubules with mitotic cells were not utilized. Stage group V–VI was identified by the presence of type B spermatogonia and step 5 and step 6 spermatids (the step 5 and step 6 spermatids were used as criteria only when they were present). Tubules at these stages containing mitotic cells were not utilized. Stage group VII–VIII was identified by the presence of preleptotene spermatocytes and step 7 and step 8 spermatids (the step 7–8 spermatids were used as criteria only when they were present). Stage group IX–XIII was distinguished by the presence of type A spermatogonia, in the absence of intermediate spermatogonia, type B spermatogonia, and preleptotene spermatocytes, and by the presence of step 9–13 spermatids (only when present). The formula presented in El Shennawy et al (1998; this volume) was used to calculate the total number of germ cells present in each stage group per hour of their development.

Data Analysis

Data analysis was conducted using the methodology presented in El Shennawy et al (1998; this volume). The critical value for significance was $P = 0.05$.

Results

Body Weights, Testis Weights, and Seminiferous Tubule Diameters

Table 1 gives body and testis weights and seminiferous diameter measurements for the two groups used in the present study. The body weights of flutamide-treated Hx animals were significantly lower than those for animals undergoing Hx without flutamide. Similarly, the testis weights were significantly lower in the Hx-flutamide group. The tubular diameter was not significantly different in the two groups. The total volume of the seminiferous epithelium was significantly decreased in the flutamide-treated group as compared with the Hx group.

Germ-Cell Numbers

The germ-cell population profile of Hx rats is shown in Figure 1. Although this technically should be viewed in bar graphs, there are reasons why line graphs were con-

Table 1. *Basic parameters (mean ± SE)*

Parameters	Hx for 36 days	Hx for 36 days + flutamide
Body weight (g)	182.9 ± 1.9*	149.5 ± 2.9
Paired testis weight (g)	0.634 ± 0.06*	0.429 ± 0.06
Tubular diameter (μm)	120 ± 2.7	109 ± 4.7
Seminiferous epithelium (Vv%)	74.6 ± 1.7	75.2 ± 1.3
Vacuoles (Vv%)	4.1 ± 1.3	1.3 ± 0.5
Lumen (Vv%)	1.3 ± 0.3	0.7 ± 0.1
Tunica propria (Vv%)	4.1 ± 0.3	4.8 ± 1.2
Intertubular space (Vv%)	15.9 ± 1.9	18.0 ± 1.2
Seminiferous epithelium volume (ml)	0.223 ± 0.024*	0.146 ± 0.024

Hx, hypophysectomy.
* Statistically significant ($P < 0.05$).

structed. Spermatogenesis in the Hx and the Hx-flutamide groups is most likely end-stage in that it takes place after long-term treatment and, thus, one can reasonably believe that the rate of cell depletion will not differ with time, since a steady state is achieved. In a steady-state process, line graphs can be used to depict temporal sequences, although animals were not sacrificed serially. We suggest that their primary use be to compare Hx and Hx-flutamide groups with one another. Finally, line graphs are more suitable for visual portrayal of the data. The greatest number of cells of any one type present within the seminiferous epithelium, as expressed per hour of development for any one stage grouping in the Hx rat, belonged to the preleptotene spermatocyte population. A progressive decline in germ-cell numbers occurred as preleptotene cells progressed to meiosis (metaphase through telophase), where an approximate doubling in cell populations occurred from late pachytene precursor cells to form young round spermatids in stage group II–IV. At stage group VII–VIII, there was a sharp decline in the step 7–8 spermatid population of cells that continued until stage grouping 9–13, where there were few cells in this

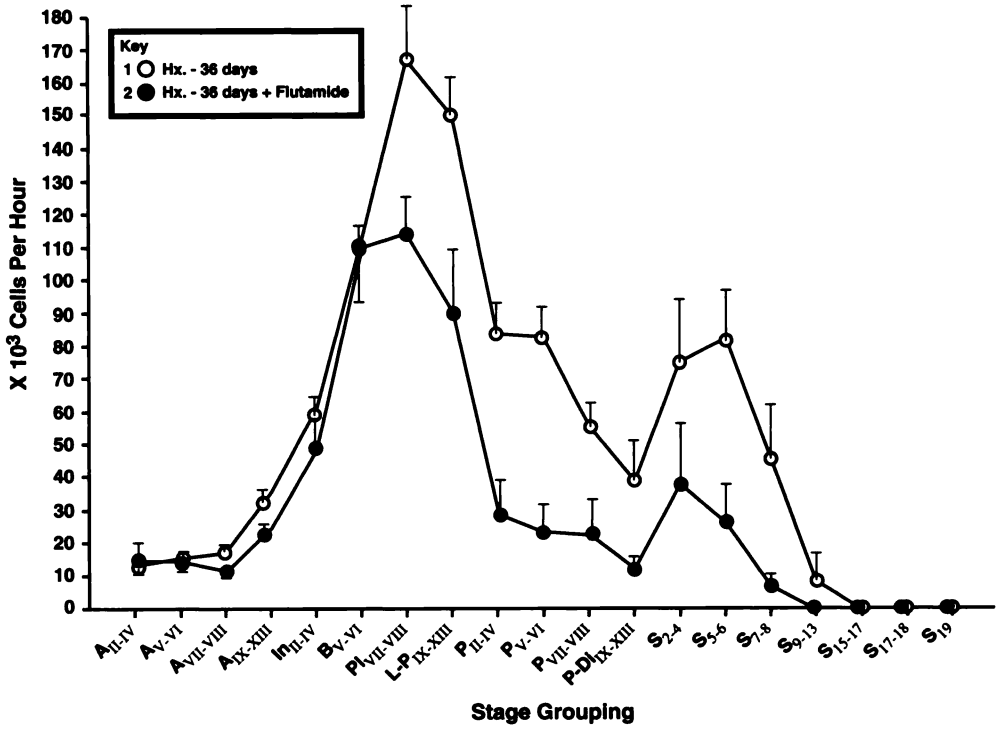

FIG. 1. Graph showing the population of germ cells from type A spermatogonia to spermatids in four stage groupings comprising the cycle of the seminiferous epithelium of the rat. The numbers of germ cells are expressed per hour of development. Abbreviations used are as follows: A, In, and B for type A, intermediate, and type B spermatogonia, respectively; Pl for preleptotene, L-P for leptotene through pachytene, P for pachytene, and P-Di for pachytene through diplotene; S_{2-4}, S_{5-6}, S_{7-8}, S_{9-13}, S_{15-17}, S_{17-18}, and S_{19} for steps of spermiogenesis, respectively. Roman numerals indicate the stage that various cell types are seen.

category present. Since steps of spermiogenesis can be recognized, the actual most advanced cell type seen in the Hx group was the step 10 spermatid. In the Hx-flutamide group, the most advanced cell type seen was the step 8 spermatid. No spermatids in the step 15–17 group were found.

Figure 1 also is a line graph of the cell populations present per hour of development in the Hx-flutamide–treated group. An overall comparison of the lines in Figure 1 shows that the Hx group had considerably more cells than the Hx-flutamide group. The reduction of cells in the Hx-flutamide group, as compared with the Hx group, was first clearly noticeable at the type B spermatogonia phase to preleptotene phase. A diminished germ-cell population in the Hx-flutamide group was evident throughout the remainder of spermatogenesis. The most mature cells seen in the treated animals were the step 7–8 spermatid group.

The percent change in germ-cell populations between one phase or stage grouping of spermatogenesis and another is provided in Figure 2. Significantly greater percent decreases in cell populations were first noted in the Hx-flutamide group, as compared with the Hx group, from stage group V–VI, when the yield of preleptotene spermatocytes was compared with the yield of their progenitor type B spermatogonia, and from stage group leptotene–zygotene (L–Z), when preleptotene spermatocytes were compared to their progenitor preleptotene cells. Similarly, there was significantly greater loss of cells in the transition from L–Z to form pachytene spermatocytes. Ironically, a significantly greater number of stage VII–VIII spermatocytes were formed from their progenitor pachytene spermatocytes (no cell division) in the Hx-flutamide group as compared with the Hx group. However, in the next phase of development, significantly greater cell loss was evident in midcycle pachytene spermatocytes as these cells developed into pachytene/diplotene cells. Significantly greater cell loss was evident in the Hx-flutamide group as spermatids matured from S_{2-4} to S_{5-6} spermatids. If one considers cells more mature than type A spermatogonia, the mean loss was greater numerically in the Hx-flutamide group as compared with the Hx group in eight of ten cell maturation phases that were compared.

Discussion

The Hx Animal

The present study has provided a quantitation of germ-cell populations during the spermatogenic cycle in long-term (36 days) Hx rats. It was shown that after about 25 days of Hx the numbers of germ cells in the testis stabilized, and no further loss occurred (Clermont and Morgentaler, 1955). Thus, what occurred from around 25 days until 36 days represented an end-stage condition in which deterioration of the testis was maximal.

The rise and fall in the population of spermatogonia followed a pattern during the spermatogenic cycle similar to that shown for the normal rat (El Shennawy et al, 1998; this volume). The population increased as differentiated spermatogonial cells (A_1 through A_4) proliferated at the end of the spermatogenic cycle. Although the pattern did not differ from the normal animal, the difference in the size of the population was great when Hx and normal animals were compared. Hypophysectomy depressed the number of spermatogonial cells by about 40%, as compared with normal animals, in one study of 44 days of Hx duration (Clermont and Morgentaler, 1955) and by about 20% in another study after 13 days of Hx (El Shennawy et al, 1998; this volume).

Cell counts in the Hx animals reflected the expected doubling of the population of intermediate spermatogonia. The conversion of type B spermatogonia to preleptotene spermatocytes was inefficient in the Hx animals as compared with the normal animal, a feature also demonstrated in the intermediate-term Hx rat (El Shennawy et al, 1998; this volume). This feature was not seen in a previous study (Clermont and Morgentaler, 1955), but it is clear from simple visual examination of the testis that many preleptotene cells were degenerating in the present study. Only recently have the morphological characteristics of degenerating preleptotene cells been identified (Ghosh et al, 1991). A comparison of cell numbers in Figure 1 herein with cell numbers in Figure 3 of El Shennawy et al (1998; this issue) showed that the normal adult testis contains over 45 times more preleptotene spermatocytes than the long-term Hx rat testis.

Preleptotene cells comprised the largest number of cells in the seminiferous epithelium for any single stage grouping. Subsequent to the preleptotene phase, there was a sharp decline in the numbers of cells until late pachytene/diakinesis. This decline was not noted in the normal animal (El Shennawy et al, 1998; this volume). During meiosis, when a theoretical quadrupling of cells occurs, an approximately twofold increase was realized in the Hx animal.

A sharp drop in cell numbers was noted during mid-spermiogenesis that effectively eliminated all spermatids at step 15–17 of spermiogenesis. This finding is in close, but not precise, agreement with past studies that found no spermatids past step 7 of spermiogenesis within the epithelium (Clermont and Morgentaler, 1955).

The presence of germ cells in Hx animals, albeit in lower numbers than in normal animals, indicated that germ-cell survival is both qualitatively and quantitatively affected (Clermont and Morgentaler, 1955). This has been recognized in previous studies, although a precise quantitation was not undertaken. In addition, the observation that the Hx animal secreted low levels of T (Sharpe,

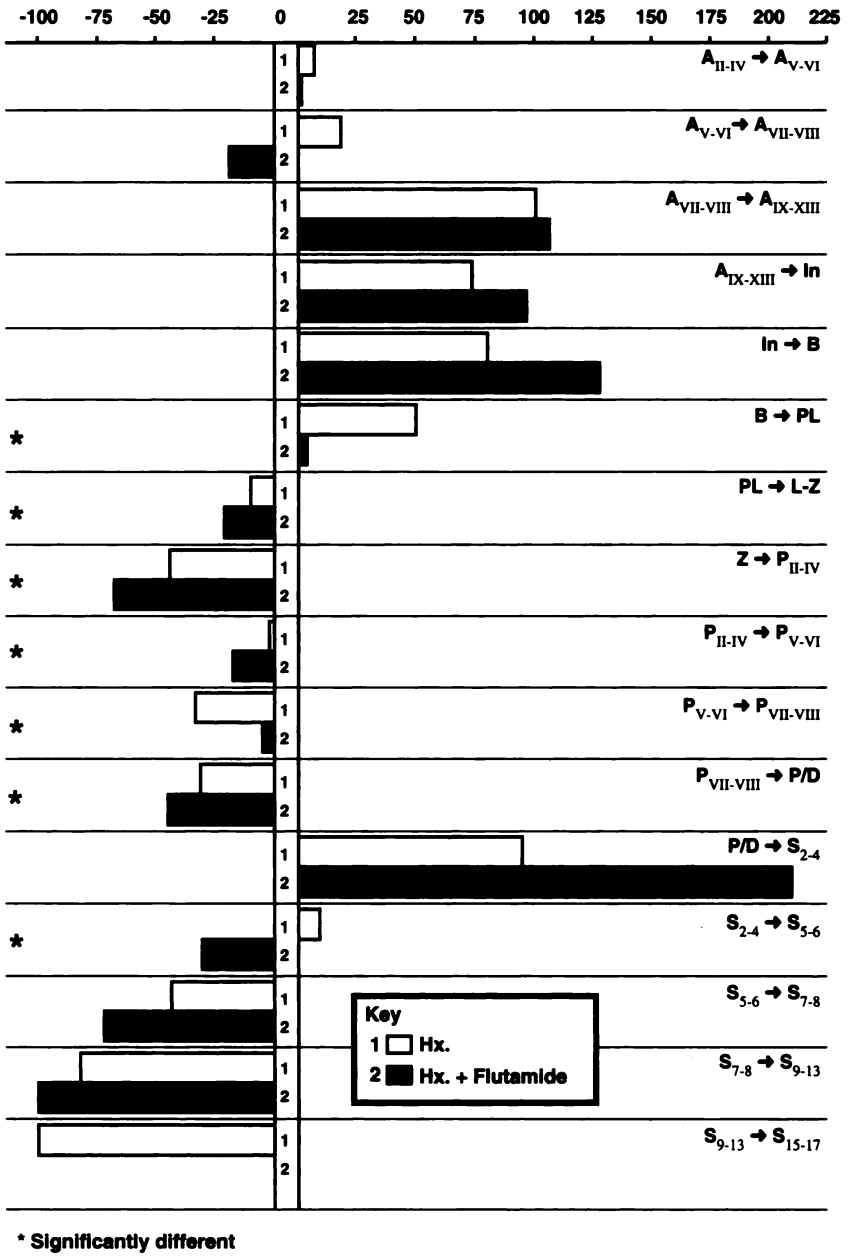

FIG. 2. Percent changes in cell populations of spermatogonia through the spermatid phases of development. An asterisk to the left of the figure indicates significant differences between the Hx and the Hx-flutamide–treated groups. The abbreviations used are the same as those detailed in the legend for Figure 1.

1994) has, to some extent, drawn into question previous studies examining hormonal control of spermatogenesis that were conducted with the assumption that T was not secreted. This provided the rationale for using an androgen inhibitor to study the specific effect of T.

The Hx-Flutamide–Treated Rats

Although there were minor (not significant) depressions in the population of differentiating type A spermatogonia in Hx animals given flutamide, these differences were largely made up for by the time that a type B spermatogonial population was attained. Given that the levels of spermatogonial degeneration were high and that the spermatogonia appeared to self-regulate their population, it could be suggested that a mechanism other than hormonal control of pituitary origin can also influence the type A spermatogonial population. Such a mechanism has been proposed and has been termed density-dependent degeneration (de Rooij and Lok, 1987). This mechanism may have been operative in the present study.

The most dramatic finding in the present study related to the inability of the population of type B spermatogonia to successfully convert to preleptotene spermatocytes in the presence of flutamide. Whereas the increase in the cell population after this division in Hx animals was 50%, the population increase in the Hx-flutamide–treated group was only about 5%. This finding suggested that selective elimination of T has pronounced effects that result in a loss of approximately 50% of the preleptotene spermatocytes, as compared with Hx animals not receiving flutamide. Hormone-sensitive loss at the preleptotene phase of spermatogenesis also was shown for adult rats after intermediate-terms of Hx (El Shennawy et al, 1998; this volume) and for adult rats after short-term Hx (Russell and Clermont, 1977). This was not the major phase of cell loss in the study by El Shennaway et al (1998) where it was shown that the production of type B spermatogonia from intermediate spermatogonia was more sensitive than the loss of preleptotene spermatocytes (Russell and Clermont, 1977). Although subsequent phases of spermatogenesis were affected by T in the present study, the blockage at preleptotene had the greatest effect on cell populations during the remainder of spermatogenesis.

That several phases subsequent to preleptotene were affected through late pachytene indicated that T deficiency does not simply impact one point in the spermatogenic cycle but has widespread effects. An approximately 200% increase in cells occurred from the pachytene/diplotene phase of spermatogenesis to the step 2–4 spermatid phase of spermatogenesis in the Hx-flutamide group. This increase was due to variability in cell counts and was not statistically significant. Selective T influence was not evident as meiotic division occurred (pachytene/diplotene forming young round spermatids) but was evident in spermatids at midcycle as the conversion of step 2–4 spermatids occurred to form step 5–6 spermatids.

Although cell degeneration was not restricted to one phase of spermatogenesis, significant cell loss occurred during, shortly before, and shortly after midcycle. The T-related loss of preleptotene spermatocytes in stages VII and VIII and the loss in step 2–6 spermatids had a major quantitative impact on the overall cell population present within seminiferous tubules.

In summary, this study used quantitative analysis to show that the germ-cell population in long-term Hx rats and long-term Hx rats receiving the androgen antagonist flutamide substantially reduced cell numbers as compared with animals not receiving flutamide. The cellular sites vulnerable to further deprivation of androgen were primarily those near, but not restricted to, midcycle.

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