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A Quantitative Study of Spermatogenesis in the Developing Rat Testis

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ABSTRACT

Quantitative (stereological) studies were performed to determine the number of germ cells in the developing rat testis. Sprague-Dawley rats aged 1–70 days were fixed by immersion or perfusion and embedded in Epon Araldite. Blocks of tissue were sectioned at 1.5 μm and stained with toluidine blue dye. Sections were systematically scanned and the areal density of nuclear profiles counted using an unbiased counting frame. Numerical density and absolute number of germ cells in the processed block were then estimated. Corrections for processing shrinkage were determined by comparing the volume of processed and unprocessed samples. The results demonstrate the necessity of determining absolute number rather than volume density (or areal density) in comparing germ cell numbers. In these experiments, spermatogonial numbers stabilized in the range 18.4–23.6 million per testis on Day 30. The number of primary spermatocytes that were first apparent on Day 15 increased rapidly to 54.6 million per testis on Day 30 and then slowly to 73.6 million on Day 70. Round spermatids were first apparent on Day 25 and increased rapidly to 85.7 million per testis on Day 40, then continued to increase to 151.9 million on Day 70. The study provides both methods and baseline data for future experiments involving manipulation of the spermatogenic potential of the testis.

INTRODUCTION

The steps in the complex process of spermatogenesis have held a fascination for biologists, and many cytological details have been elucidated (see review de Kretser and Kerr [1]). Many of the earlier quantitative studies of spermatogenesis in the adult rat testis have been complemented by recent morphometric data from the detailed study of Wing and Christensen [2]. Despite these quantitative studies on the adult testis, little attention has been paid to the developmental process in rats despite several hormonal studies during sexual maturation (see review, Ojeda and Urbanski [3]). Recently, morphometric techniques have been applied to the estimation of Sertoli cell numbers in the rats, establishing that no significant changes occurred beyond 20 days [4]. The present study was undertaken to quantitatively characterize the major changes in germ cells in the developing rat testis using contemporary morphometric techniques.

MATERIALS AND METHODS

Animals

Groups of 4–5 male Sprague-Dawley rats, aged 1, 5, 10, 15, 20, 25, 30, 35, 40, 48, 60, and 70 days, maintained on a constant light cycle (14 L:10 D) were obtained from the Central Animal House, Monash University, Clayton, Victoria, Australia. They were fed ad libitum before the experimental procedures began.

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Fixation and Processing

Animals aged 1, 5, 10, and 15 days were anesthetized by inhalation of ether and the testes were removed and weighed. Testis density was determined by immersion in graded sucrose solutions of known density [5], and volume was calculated. The testes were then immersed in fixative (5% glutaraldehyde, 4% formaldehyde, and 0.05% 2,4,6-trinitrocresol buffered to pH 7.4 with 0.2M cacodylate [6]). After fixation for 3–4 h, the right testis was cut into a series of parallel slices orthogonal to its long axis. In the case of the 1-day-old rat, the testis was divided into 2, and both halves were processed; the 5- and 10-day testes were divided into 4 slices, and a central slice, together with the 2 ends, were processed; the 15-day testis was divided into 5 slices, and central and end slices were taken for processing. The slices were kept in fixative overnight at 4°C, postfixed in osmium, and processed into Epon-Araldite [7]. Sections were then cut on a Reichert OMU3 microtome at 1.5 μm and stained with Toluidine blue dye; sectioned material from the immersion-fixed testis was of comparable quality to that obtained by perfusion when examined by light microscopy. However, immersion fixation for testes from older animals is generally inferior to perfusion.

Animals aged 20, 25, 30, 35, 40, 48, 60, and 70 days were injected i.m. with heparin (Heparin BP injection, 5000 units/ml, Weddel Pharmaceuticals Ltd., London, UK) (15 units/g body weight). The animals were anesthetized by ether inhalation and perfused for 15–20 min via the thoracic aorta with the same fixative as the younger animals. The testes were removed and weighed, and the weight was transformed to a volume by correcting for the density of the perfused testes at that age; 1-mm-thick slices were then cut orthogonal to the long axis from the top, middle, and bottom. The slices were further subdivided into 3 × 3-mm

blocks and processed and sectioned as described above; one block from each slice was sectioned. The right testis was used for stereological studies.

Tissue Shrinkage

Shrinkage during processing was assessed by a variation of the "Fractionator" method described by Gundersen [8]. After determining the volume of the fresh testis in the case of younger animals and the perfused testis in the case of older animals, a block representing a known fraction of the testis was determined by weight using a Mettler AE166 balance with a reproducibility of 0.1 mg (in the case of 1-day-old animals, the whole testis was used). This block was then processed as described above and serially sectioned at 1.5 or 2.5 μm. The total length of the block orthogonal to the section plane was determined before and after exhaustive sectioning, using a metric micrometer (Moore and Wright, Sheffield, UK), and the average section thickness was calculated by dividing the difference in length of the block by the number of sections cut. Depending on the size of the block, between 1 in 20 and 1 in 60 sections were selected for area measurement (between 15 and 30 sections were measured in each block) by point counting, using a projecting microscope (Leitz Neopromar) at a final magnification of 26× with larger blocks and 65× with smaller blocks. A coherent square grid with points at 5 cm and 2.5 cm was used for area estimation. The volume of the processed block was then calculated using Cavalieri's principle [9].

Section Compression

Compression of the sections was assessed by photographing both a stained section and the block face through a stereomicroscope. The final magnification of the micrographs was 21.3. Dimensions in both the cutting direction and a direction at right angles to it were assessed.

Stereological Estimation

The numbers of germ and Sertoli cells were estimated by determining the number of nuclei of each cell type and assuming a 1:1 correspondence. Germ cells were classified into spermatogonia, all classes of primary spermatocytes, and round spermatids (stages 1–7 [10]); no attempt was made to count elongated spermatids. In one-day-old animals, the only germ cells present were gonocytes; at 5 days, a mixture of gonocytes and spermatogonia were present, at 15 days, gonocytes were absent. For the purposes of calculation, gonocytes were grouped together with spermatogonia.

The number of germ cells per unit volume of testis was estimated using the De Hoff and Rhines equation [11] with corrections for section thickness [12] and lost caps [13]:

$$N_v = \frac{N_A}{(\bar{D} + t - 2h)}$$

where N_v = Numerical density of the nuclei
N_A = Areal density of the nuclear profiles
$\bar{D}$ = the mean caliper diameter
t = the section thickness
and h = the height of the lost caps.

N_A was determined using a 40× oil immersion lens (Nikon Plan Apo NA 1.0) in conjunction with a 10× eyepiece fitted with a graticule marked with a coherent test grid consisting of a double-square lattice with a distance between course points of 41 μm and a distance between fine points of 20.5 μm (at this magnification). Using this graticule, an unbiased counting frame [14] was defined, and all profiles of a particular cell type falling within the frame were counted. The area within which profiles had been counted was estimated by counting the number of central points within each square of the lattice that hit the seminiferous tubule (seminiferous epithelium and lumen); the area of interstitium was also estimated at this stage. In the case of 1-, 5-, and 10-day-old animals, the whole of the area included in the graticule (411 μm × 411 μm) falling on a single field was counted in each section. In the case of 15-day-old animals, half of the area of the graticule was counted on 2 fields located diagonally across the section. In the case of 20–70-day-old animals, an area measuring 164 × 164 μm in the upper left hand corner of the graticule was sampled. The sampling frame was moved across the sections starting in the upper left-hand corner and progressing across the slide in 0.5-mm increments; additional parallel strips (0.5 mm apart) were sampled until 10 fields had been counted per section.

The mean caliper diameter of the germ cells was estimated by measuring the profile diameters of nuclei. Germ cell nuclear profiles were slightly elliptical, the ratio of long-to-short axes being in the range 0.80–0.85. Accordingly, the mean of the long and short axes was taken as the profile diameter. The diameter of 300 systematically sampled profiles (sampled as for N_A determination) was measured, using a Leitz ASM image analysis system in conjunction with a 63× Leitz oil immersion objective (numeric aperture 1.3). The results were plotted on a Wicksell-type [15] histogram of number versus profile diameter at 1-μm intervals from 0–14 μm. The histogram was then corrected for missing profiles by extrapolating back in zero diameter, and the corrected diameter was determined using the method of Giger and Riedwyl [16] as summarized in Weibel [17]. The value of the lost cap correction (h) could be determined from the same histogram and was consistently equal to D/14.5.

The section thickness was determined on a number of occasions in the blocks used to determine shrinkage.

As a further check on the diameter of the germ cells, serial sections were cut as for Sertoli cells, and the diameter of the largest profile of each nucleus sampled, using a "Disector" [18] was measured.

The volume of Sertoli cell nuclei in testes from rats aged

1–20 days was estimated using the point-sampled intercept described by Gundersen and Jensen [19] and used by Wang *et al.* [4] to estimate the number of Sertoli cells in the testis. Sections were placed on the microscope stage and centered using a 4× lens. The lens was then changed to a 40× oil immersion (Nikon Plan Apo NA 1.0), and the two tubules closest to the center of the field were photographed; photomicrographs were printed at a final magnification of 1842×. Points for sampling were then selected with a 1.2-cm square grid in the case of 1-day-old animals and a 1.5-cm square grid in the case of older animals. Intercepts were classified on a linear scale [20], and the mean volume in the volume distribution was estimated using the equation

$$\bar{v}_V = \frac{\pi}{3}\overline{l_{oi}^3}$$

where $\bar{v}_V$ = the volume-weighted mean volume of the nuclei and l_{oi} = the length of the point-sampled intercept through the i^{th} point. Between 80 and 100 intercepts were measured in each animal. The volume fraction of Sertoli cell nuclei in seminiferous tubules was estimated on the micrographs by point counting using the same grid used for selecting point-sampled intercepts.

The number of Sertoli cell nuclei was then estimated using the equation:

$$N_{scn,t} = \frac{V_{Vscn,st} \cdot V_{Vst,t} \cdot V_t}{\bar{v}_{V,scn}}$$

where $N_{scn,t}$ = the number of Sertoli cell nuclei per testis
$V_{Vscn,st}$ = the volume fraction of Sertoli cell nuclei in the seminiferous tubule
$V_{Vst,t}$ = the volume fraction of seminiferous tubules in the testis
V_t = the reference volume of the testis corrected for shrinkage during processing
$\bar{v}_{V,scn}$ = the mean volume of Sertoli cell nuclei in the volume-weighted distribution of volume.

Since the assumption that $\bar{v}_V$ equals $\bar{v}_N$ (mean volume in the number-weighted distribution) for Sertoli cell nuclei has never been proven, $\bar{v}_N$ was estimated in a few animals (1, 10, 20, and 70 days) using the "Selector" described by Cruz-Orive [20]. A serial stack of 10–20 sections was cut at 1.5 μm. A tubule was selected at random in the first section and then photographed (printed at 1842×) on each of the succeeding sections. Cells were selected for measurement by comparing sequential sections. If a nucleus was present in the second section but absent in the first (the "disector" method [18]), it was then followed throughout the serial stack until no further profiles were cut. The mean of the cube of the point-sampled intercepts over that nucleus was then averaged to give an estimate of the volume of the nucleus; between 20 and 43 nuclei were sampled in each case. After estimation of both $\bar{v}_V$ and $\bar{v}_N$ in these cases, an estimate of the coefficient of variation of $\bar{v}_N$,($CV_N(\bar{v})$) can be calculated from the equation [20]:

$$CV_N^2(\bar{v}) = \frac{\bar{v}_V}{\bar{v}_N} - 1$$

Statistics

All data in text, tables, and figures are shown as mean ± standard deviation. Statistical comparison between groups is by analysis of variance in conjunction with Duncan's multiple-range test.

RESULTS

Volume and Density

The testis volume increased steadily with age (Table 1) with the exception of testes from 15- and 20-day animals, which did not differ significantly from each other for unknown reasons. The density of the testes showed little age dependence; the major influence on density was the fixation procedure. The density of fresh testes in 1–15-day-old rats was 1.046 ± 0.003, whereas that of perfusion-fixed testes in 20–70-day-old rats was 1.040 ± 0.002. The volume fraction of seminiferous tubules in the testes increased by 2.5-fold from Day 1 to Day 30 and showed little variation thereafter. The absolute volume of seminiferous tubules increased 155-fold from Day 1 to Day 30 and 5-fold from Day 30 to Day 70.

Tissue Shrinkage and Compression

Shrinkage in the immersion-fixed testes varied with the age of the animal. Processed volume as a fraction of fresh volume was 0.756 ± 0.011 at 1 day and 0.761 ± 0.041 at 5 days. At 10 and 15 days, the processed volume as a fraction of fresh volume was 0.852 ± 0.034 and 0.846 ± 0.010, re-

TABLE 1. Volume and volume fraction data for the right testis of rats aged from 1–70 days.

Age (days)	V[a] ± SD (mm³)	$V_{Vst,t}$[b] ± SD	$V_{st,t}$[c] ± SD (mm³)
1	4.5 ± 0.7	0.358 ± 0.036	1.6 ± 0.3
5	12.0 ± 0.3	0.437 ± 0.027	5.2 ± 0.3
10	25.9 ± 3.0	0.556 ± 0.038	14.4 ± 1.7
15	56.7 ± 6.0	0.654 ± 0.049	37.1 ± 5.1
20	58.0 ± 1.0	0.851 ± 0.035	49.0 ± 3.4
25	150 ± 5	0.871 ± 0.056	130 ± 1
30	278 ± 33	0.900 ± 0.034	248 ± 49
35	424 ± 18	0.904 ± 0.040	392 ± 54
40	645 ± 21	0.874 ± 0.046	563 ± 10
48	725 ± 95	0.895 ± 0.029	655 ± 182
60	1015 ± 95	0.912 ± 0.033	927 ± 182
70	1281 ± 38	0.898 ± 0.008	1150 ± 73

[a] V = Volume of testis.
[b] $V_{st,t}$ = Volume fraction of seminiferous tubule in testis.
[c] $V_{st,t}$ = Volume of seminiferous tubule in testis.

spectively. At 20, 40, and 70 days, the processed volume as a fraction of the perfusion-fixed volume was 0.867 ± 0.022, 0.888 ± 0.057, and 0.883 ± 0.027, respectively. Final corrections were based on the grouped data, i.e. 0.759 for 1- and 5-day-old rats, 0.849 for 10- and 15-day-old rats, and 0.880 for 20–70-day-old rats.

Section compression was negligible in sections cut at 1.5 μm. When dimensions of block and section were compared, dimension changes were less than 1%, and accordingly no corrections were necessary.

An accurate estimate of section thickness is necessary for determinating processed volume of tissue blocks. Repeat estimates of section thickness using the Reichert OMU3 gave a thickness of 1.528 ± 0.004 (Mean ± SD) for nominal 1.5 μm sections. This estimate was subsequently used for the section thickness correction in numerical density determinations.

Stereological Estimates

There was little inter-animal variation in nuclear size at any age. The mean diameter of spermatogonial nuclei (Table 2) decreased from Day 1 to Day 15 corresponding to the transition of gonocytes to spermatogonia. The mean diameter of primary spermatocyte nuclei (Table 2) increased from Day 15 to Day 40, corresponding to the growth in nuclear size as these cells progressed through the stages of the first meiotic prophase. The mean diameter of round spermatids (Table 2) was constant from their first consistent appearance at Day 30 to Day 70. By use of the method of Giger and Riedwyl [16], an estimate of the standard deviation of nuclear size within animals was also obtained; all the germ cell types showed little intra-animal variation in nuclear size, the coefficient of variation averaging 8.2 ± 2.5% for spermatogonia, 8.5 ± 2.0% for spermatocytes, and 6.6 ± 2.6% for spermatids.

Reconstruction of nuclei from serial sections was employed in a few cases to check the true nuclear size. In the case of spermatogonial nuclei at 20 days (measured in a single animal), the true observed nuclear diameter was 7.05 ± 0.50 μm (n = 24), whereas that obtained by the method of Giger and Riedwyl [16] was 7.06 μm. Similarly, in the case of primary spermatocyte nuclei at 70 days, the true diameter in one animal was 10.69 ± 0.43 μm (n = 30), whereas that obtained by the method of Giger and Riedwyl [16] was 10.83 μm. In the case of round spermatid nuclei at 70 days, the true diameter was 8.67 ± 0.32 μm (n = 26), and that obtained by the method of Giger and Riedwyl [16] was 8.77 μm. The ratio of observed profile diameter to nuclear diameter after reconstruction of the full profile distribution and correcting for sectioning [16] proved to be relatively constant for all cell types at 1.18 ± 0.01.

The areal density of spermatogonia (Fig. 1) initially declined between 1 and 5 days (gonocytes were counted as spermatogonia in these animals) and then increased to peak at 2.62 ± 0.42 nuclear profiles per thousand μm^2 of tubule on Day 15 before declining to 0.18 ± 0.02 on Day 70. A similar peak (289 ± 50 nuclei per million μm^3 of tubule) was observed in the numerical density (Fig. 2) on Day 15, which then declined to 20.2 ± 1.4 nuclei per million μm^3 on Day 70. However, when the absolute number of spermatogonia (Fig. 3) was monitored, no decrease in absolute numbers was apparent due to the compensating growth of the testes. From Day 30–70, the total number of spermatogonia showed no significant variation and stayed in the range of 18.4–23.6 million per testis.

Primary spermatocytes (Fig. 1) were first apparent on Day 15, and their areal density peaked at 2.48 ± 0.36 nuclear profiles per thousand μm^2 of tubule on Day 25, declined rapidly to 1.13 ± 0.07 on Day 40, and then declined slowly to 0.85 ± 0.01 on Day 70. A similar pattern was observed with the numerical density (Fig. 2), with a maximum of 235.2 ± 40.7 per million μm^3 of tubule on Day 25 declining rapidly to 92.2 ± 6.9 on Day 40 and then slowly to 64.1 ± 1.2 on Day 70. The absolute number of primary spermatocytes (Fig. 3) showed a relatively rapid increase from Day 20–35

TABLE 2. Diameter of germ cell nuclei corrected for sectioning and missing profiles by the method of Giger and Riedwyl.

Age (days)	Mean diameter		
	Spermatogonia (μm)	Primary spermatocyte (μm)	Round spermatid (μm)
1	11.42 ± 0.39		
5	9.27 ± 0.31		
10	8.32 ± 0.13		
15	7.20 ± 0.26	8.21 ± 0.12	
20	7.00 ± 0.13	8.59 ± 0.16	
25	7.24 ± 0.10	8.99 ± 0.30	*
30	7.19 ± 0.30	9.42 ± 0.28	8.27 ± 0.41
35	6.90 ± 0.31	9.34 ± 0.09	8.27 ± 0.22
40	7.13 ± 0.21	10.25 ± 0.20	8.17 ± 0.29
48	6.98 ± 0.44	10.22 ± 0.47	8.31 ± 0.10
60	6.95 ± 0.18	10.32 ± 0.50	8.17 ± 0.18
70	7.48 ± 0.27	11.04 ± 0.16	8.41 ± 0.52

*Only a small number of round spermatids were observed at Day 25. For the purpose of subsequent calculations, the diameter was assumed to be the same as that at Day 30.

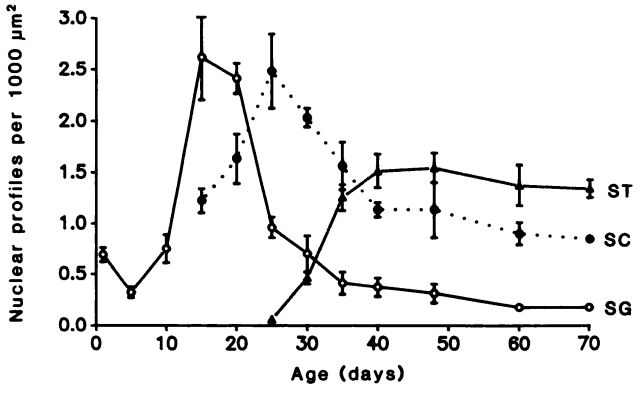

FIG. 1. The number (Mean ± SD) of germ cell profiles per thousand μm^2 of seminiferous tubule (areal density) as a function of age. SG, spermatogonia; SC, spermatocyte ST, spermatid.

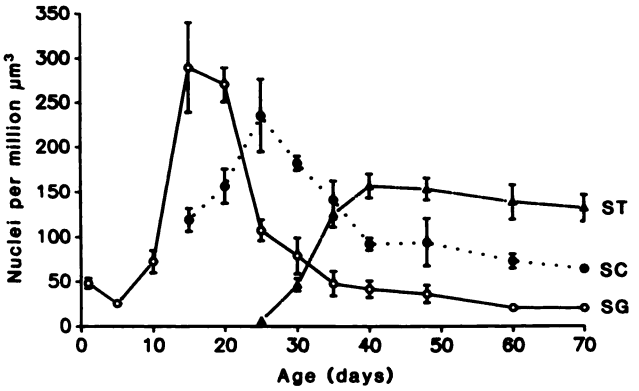

FIG. 2. The number (Mean ± SD) of germ cells per million μm^3 of seminiferous tubule (numerical density) as a function of age. The reference volume is fresh testes for animals 1–15 days old and fixed testes for older animals. SG, spermatogonia; SC, spermatocyte; ST, spermatid.

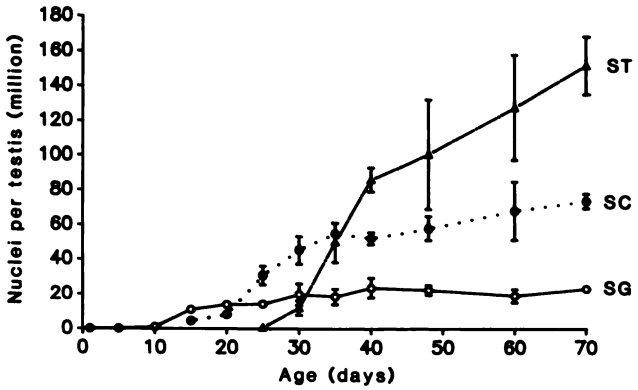

FIG. 3. The number (Mean ± SD) of germ cells per testis as a function of age. SG, spermatogonia; SC, spermatocyte; ST, spermatid.

(8.0 ± 1.2 to 54.6 ± 6.1 million per testis) and then a slow increase to 73.6 ± 4.2 million per testis on Day 70. There was no significant difference between the number of spermatocytes on Days 48, 60, and 70.

Round spermatids were first observed on Day 25 (3 of 4 animals), and their areal density (Fig. 1) peaked between Day 40 and Day 48 (1.51 ± 0.16 and 1.64 ± 0.14 nuclear profiles per thousand μm^2 of tubule respectively) before decreasing slightly to 1.34 ± 0.09 on Day 70. A similar pattern was observed with the numerical density (Fig. 2), which peaked between Day 40 and Day 48 (156.7 ± 13.4 and 153.2 ± 12.3 nuclei per million μm^3 of tubule, respectively) and then decreased slightly to 132.2 ± 15.1 on Day 70. However, the absolute number (Fig. 3) increased rapidly between Day 25 and Day 40 (85.7 ± 7.0 million per testes) and then continued to increase to 151.9 ± 16.7 million per testis at Day 70 (there was no significant change between Day 48 and Day 70).

The volume-weighted mean volume of Sertoli cell nuclei decreased from 202 ± 33 μm^3 at Day 1 to 136 ± 12 μm^3 at Day 10 and then increased to 173 ± 23 μm^3 at Day 20 (Table 3). At the same time, the volume fraction of Sertoli

TABLE 3. Estimation of the number of Sertoli cells in the right testis.

Age (days)	$\bar{v}_V$[a] (μm^3)	$V_{Vscn,st}$[b]	N[c] (million)
1	202 ± 33	0.246 ± 0.017	1.5 ± 0.4
5	153 ± 14	0.237 ± 0.011	6.2 ± 0.8
10	136 ± 12	0.261 ± 0.033	23.6 ± 3.6
15	166 ± 20	0.156 ± 0.012	29.9 ± 4.5
20	173 ± 23	0.118 ± 0.011	30.0 ± 2.5

[a] $\bar{v}_V$ = Volume-weighted mean volume of Sertoli cell nuclei.
[b] $V_{Vscn,st}$ = Volume fraction of Sertoli cell nuclei in the seminiferous tubule.
[c] N = Absolute number of Sertoli cells in the right testis.

cell nuclei in the seminiferous tubules decreased from 0.246 ± 0.017 to 0.118 ± 0.011 (Table 3); however, this decrease was compensated by a rapid increase in the total volume of seminiferous tubules from 1.61 to 49.0 mm^3 (Table 1) in the same period. Consequently, the overall number of Sertoli cell nuclei increased from 1.5 ± 0.4 on Day 1 to 30.0 ± 2.5 million per testis on Day 20.

The volume-weighted mean volume of Sertoli cell nuclei was compared with the number-weighted mean volume obtained using the "Selector" [20] in selected cases (Table 4). In no case did an error greater than 10% arise from assuming the equality of volumes in number and volume distribution.

DISCUSSION

This study provides quantitative data for the important developmental changes in the spermatogenic process during sexual maturation in the rat and provides the foundation for experimental manipulation of this process. It documents the changes in germ cell numbers in the major cell types to the round spermatid stage and explores the use of several stereological techniques to achieve this aim.

A confounding problem in the estimation of cell number in a tissue is the relationship between the processed volume of the tissue and the original measured reference volume. Bertram et al. [21] have demonstrated in the liver that shrinkage is not homogeneous; different components of the block shrink or swell depending on tissue type. In this study, a modification of the "Fractionator" [8] has been used to

TABLE 4. Comparison of the volume weighted and number weighted-mean volume of Sertoli cell nuclei in selected animals.

Age (days)	$\bar{v}_V$[a] (μm^3)	n_1[b]	$\bar{v}_N$[c] (μm^3)	n_2[d]	$\bar{v}_V/\bar{v}_N$	$CV_N(v)$[e]
1	184	116	168	20	1.10	.309
10	168	169	160	39	1.05	.223
20	191	57	189	43	1.01	.103
70	624	118	585	23	1.07	.258

[a] $\bar{v}_V$ = Volume weighted mean volume of Sertoli cell nuclei.
[b] n_1 = The number of point sampled intercepts used to determine $\bar{v}_V$.
[c] $\bar{v}_N$ = Number weighted mean volume of Sertoli cell nuclei.
[d] n_2 = The number of nuclei that were serially sectioned to determine $\bar{v}_N$.
[e] $CV_N(v)$ = The coefficient of variation of volume in the number distribution.

determine tissue shrinkage during processing. Testes from younger rats shrink more than those from older rats. There is a significant difference ($p < 0.01$) between shrinkage of testes of young rats (1 and 5 days) and of older rats (10 and 15 days). There is a small difference between the shrinkage observed in the testes at 10 and 15 days and that observed at 20, 40, and 70 days ($p < 0.05$).

Wing and Christensen [2] estimated volume shrinkage of perfused tissue during processing into Epon-Araldite to be 54.12% (processed volume 45.88% of original volume). Hardy et al. [22], in their elegant study on Leydig cell development, corrected their stereological data for shrinkage but did not report the actual data. In both of these studies, shrinkage was determined as the cube of a reduction in one or two linear dimensions of the block. In this study, volume shrinkage has been determined directly from the volumes of the unprocessed and the processed tissue. Given that processing in all three studies is similar, it is difficult to account for the discrepancy between the overall shrinkage.

Section compression is a possible source of distortion in the estimation of cell number. In this study using 1.5- and 2.5-μm sections, compression estimated by direct measurement was always less than 1%, and this was consistent with the observation of Bertram et al. [21], who found compression in Epon-embedded goat lung to be between 1% and 3%, and with Wing and Christensen [2], who found compression in Epon-Araldite-embedded testes to be about 1%.

Section thickness correction is often equated with the correction for lost caps and then ignored [17]. In this study, section thickness has been accurately determined by cutting a large number of sections at a given thickness and measuring the total thickness sectioned. The method gives an accurate estimate (within 1%) of the final section thickness in the absence of significant compression. Correction for lost caps is best estimated from the histogram for particle-size distribution. In this study, the height of lost caps was consistently 1/14.5 of the mean nuclear diameter; this compares with the widely used but arbitrary estimate of 1/10 suggested by Mori and Christensen [23].

Estimation of the mean caliper diameter of nuclei is probably the most difficult problem in using the de Hoff and Rhines equation [11]. The relationship between mean caliper diameter and mean profile diameter is well defined for uniform spheres having perfect contrast with the surrounding cytoplasm. The correction for lost profiles described by Giger and Riedwyl [16] is best confirmed by serial sectioning and measuring the diameter of the largest profile. Care must be taken that the profiles for this latter measurement are selected in the number distribution by the use of the "Disector" [18] to avoid biasing the nuclear diameter by its height (when nuclei are not uniform). In this study, the comparison of the mean caliper diameters determined by the Giger and Riedwyl method [16] and that obtained from serial sectioning were very close, and there was no point in repeating the comparison for all animals sampled. The ratio of observed profile diameter to true diameter was between 1.13 and 1.20 with a mean of 1.18 and a standard deviation of 0.01. There was no consistent variation in this ratio over the range of nuclear size examined, and in future studies of the same system, it could be safely assumed as 1.18.

With respect to the number of germ cells, it is apparent that changes in estimators of areal and numerical density need to be carefully examined in the light of growth of the testis. Examination of Figures 1, 2, and 3 demonstrates the potential for misinterpretation of data when the reference volume of the testis is ignored. The strikingly lower coefficient of variation in density estimates compared to absolute number suggest that control of germ cell proliferation in the testis is more regulated than control of testis size.

Wing and Christensen [2] estimated the number of Sertoli cells in adult rat testis to be 21.9 million. Their data were corrected for shrinkage using a factor of 0.4588 compared to 0.880 in the present study. If the shrinkage factor measured in this study is applied to their results, an estimate of 42 million Sertoli cells per testis is obtained. Wang et al. [4] determined the number of Sertoli cells in the testis to be constant from 15 days at approximately 38 million assuming that the processed volume of the testis was equal to the original volume. Given that the processing of testes was the same as in the present study, this would correspond to 33.4 million after correction for shrinkage. In this study, the testis in 20-day-old rats contained 30.0 ± 2.5 million Sertoli cells, which is not significantly different from the earlier study. In the earlier study [4], the equality of volume-weighted ($\bar{v}_V$) and number-weighted mean volumes ($\bar{v}_N$) of Sertoli cell nuclei was assumed. In this study, this assumption has been checked in selected cases using the "Selector" [20]. These studies indicate that an error in $\bar{v}_N$ no greater than 10% will result from this assumption. Since $\bar{v}_V$ can be estimated rapidly from single sections, it would seem to be the method of choice in future studies on the response of Sertoli cell number to experimental manipulations. When the estimates of mean Sertoli cell nuclear volume are compared with those of the earlier study, it is apparent that where similar methods are used (i.e. at 20 and 70 days), there is no significant difference in the results. When the data for animals between 1 and 15 days are compared, it is apparent that the figures in the earlier work are consistently lower than reported in this work. Given that the previous volume estimate was based on fairly crude assumptions about the mean tangent diameter, the discrepancy is not surprising.

The categorization of germ cells in this study was chosen to provide a relatively rapid assessment of the spermatogenic process during development. The grouping of spermatogonia with gonocytes at 1, 5, and 10 days reflects the difficulty of unambiguously assigning all profiles at these

stages of development in this type of preparation. Although the grouping of all primary spermatocytes into a single class may produce a small bias in the estimates of actual number, the procedure was adopted to simplify the steps necessary to obtain quantitative data. There is considerable difficulty in assigning primary spermatocytes to the subclasses of leptotene, zygotene, and pachytene stages on 1.5-μm sections since their identification depends on chromatin patterns that are hard to discern in the material used. The grouping of round spermatids at steps 1–7 again reflects the difficulty of unambiguously assigning profiles to individual stages. This categorization provides baseline data which enable quantitative techniques to be applied to experimental manipulations of the developmental process, some of which disrupt the stage-specific architecture of the testis. There is a progressive increase in spermatogonial numbers until Day 30 with no change thereafter, numbers ranging from 18.4–23.6 million/testis. The number of primary spermatocytes progressively increases to reach 73.6 million/testis at Day 70, a figure comparable to 79×10^6/testis obtained by Wing and Christensen [2] in 68-day-old rats. The round spermatid numbers reach 151.9 million/testis at Day 70 and show no evidence of plateauing.

The conversion rate of spermatogonia to primary spermatocytes as determined by the ratio of their numerical densities (Table 5) increases rapidly from Day 20 (0.59 ± 0.08) to Day 25 (2.24 ± 0.54) and thereafter shows a slow, age-dependent increase. Similarly, the conversion of primary spermatocytes to spermatids (Table 5) shows a rapid increase from Day 30 (0.26 ± 0.05) to Day 40 (1.65 ± 0.23) and thereafter a slow, age-dependent increase. The physiological basis of these dramatic increases in conversion rate requires intensive study. Some of the increase in conversion rate is likely to reflect the increased hormonal stimulation of FSH, LH, and testosterone during sexual maturation [24]. The availability of the quantitative data in this paper will enable detailed investigations of the effects of hormonal manipulations on spermatogenesis to be undertaken.

TABLE 5. Germ cell conversion ratios.

Age (days)	Sc/Sg[a]	St/Sc[b]
20	0.59 ± 0.08	
25	2.24 ± 0.54	0.02 ± 0.02
30	2.43 ± 0.54	0.26 ± 0.05
35	3.18 ± 1.02	0.90 ± 0.16
40	2.38 ± 0.84	1.65 ± 0.23
48	2.64 ± 0.22	1.71 ± 0.44
60	3.53 ± 0.53	1.96 ± 0.32
70	3.25 ± 0.21	2.04 ± 0.25

[a]Sc/Sg-Ratio of the number of primary spermatocytes to the number of spermatogonia.

[b]St/Sc-Ratio of the number of round spermatids to the number of spermatocytes.

REFERENCES

1. de Kretser DM, Kerr JB. The cytology of the testis. In: Knobil E, Neill JD (eds), The Physiology of Reproduction. New York: Raven Press; 1988: pp. 837–932.
2. Wing TY, Christensen AK. Morphometric studies on rat seminiferous tubules. Am J Anat 1982; 165:13–25.
3. Ojeda SR, Urbanski HF. Puberty in the rat. In: Knobil E, Neill JD (eds.), The Physiology of Reproduction. New York: Raven Press; 1988: pp. 1699–1738.
4. Wang ZX, Wreford NGM, de Kretser DM. Determination of Sertoli cell numbers in the developing rat testis by stereological methods. Int J Androl 1989; 12:58–64.
5. Weast RC, Astle MJ. CRC Handbook of Chemistry and Physics. Florida: CRC Press Inc.; 1980.
6. Kerr JB, de Kretser DM. Cyclic variations in Sertoli cell lipid content throughout the spermatogenic cycle in the rat. J Reprod Fert 1975; 43:1–8.
7. Kerr JB, Rich KA, de Kretser DM. Alterations in the fine structure and androgen secretion of the interstitial cells in the experimentally cryptorchid rat testis. Biol Reprod 1979; 20:409–422.
8. Gundersen HJG. Stereology of arbitrary particles: a review of unbiased number and size estimators and the presentation of some new ones, in memory of William R. Thompson. J Microsc 1986; 143:3–45.
9. Michel RP, Cruz-Orive LM. Application of the Cavalieri principle and vertical sections method to lung: estimation of volume and pleural surface area. J Microsc 1988; 150:117–36.
10. Leblond CP, Clermont Y. Definition of the stages of the cycle of the seminiferous epithelium in the rat. Ann NY Acad Sci 1952; 55:548–573.
11. De Hoff RJ, Rhines FN. Determination of the number of particles per unit volume from measurements made on random plane sections: the general cylinder and the ellipsoid. Transactions of the Metallurgical Society of AIME 1961; 221:975–982.
12. Abercrombie M. Estimation of nuclear population from microtomic sections. Anat Rec. 1946; 94:239–247.
13. Floderus S. Untersuchungen über den Bau der menschlichen Hypophyse mit besonderer Berücksichtigung der quantitativen mikromorphologischen Verhältnisse. Acta path microbiol Scand 1944; Suppl. 53:1–276.
14. Gundersen HJG. Notes on the estimation of the numerical density of arbitrary profiles: the edge effect. J Microsc 1977; III:219–223.
15. Wicksell SD. The corpuscle problem. I. Biometrika 1925; 17:84–99.
16. Giger H, Riedwyl H. Bestimmung der Grössenverteilung von Kugeln aus Schnittkreisradein Biometr Zschr 1970; 12:156.
17. Weibel ER. Stereological Methods, vol. 1. London: Academic Press; 1979.
18. Sterio DC. The unbiased estimation of number and sizes of arbitrary particles using the disector. J Microsc 1983; 134:127–136.
19. Gundersen HJG, Jensen EB. Stereological estimation of the volume-weighted mean volume of arbitrary particles observed on random sections. J Microsc 1985; 138:127–142.
20. Cruz-Orive LM. Particle number can be estimated using a disector of unknown thickness: the selector. J Microsc 1987; 145:121–142.
21. Bertram JF, Sampson PD, Bolender RP. Influence of tissue composition on the final volume of rat liver blocks prepared for electron microscopy. J Elect Micros Tech 1986; 4:303–314.
22. Hardy MP, Zirken BR, Ewing LL. Kinetic studies on the development of the adult population of Leydig cells in testes of the pubertal rat. Endocrinol 1989; 124:762–770.
23. Mori H, Christensen AK. Morphometric analyses of Leydig cells in the normal rat testes. J Cell Biol 1980; 84:340–354.
24. Lee VWK, de Kretser DM, Hudson B, Wang C. Variations in serum FSH, LH and testosterone levels in male rats from birth to sexual maturity. J Reprod Fert 1975; 42:121–126.