QUANTIFICATION OF HUMAN SEMINIFEROUS EPITHELIUM

I. HISTOLOGICAL STUDIES IN TWENTY-ONE FERTILE MEN WITH NORMAL CHROMOSOME COMPLEMENTS

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Summary. In order to provide a reference for evaluation of spermatogenesis in patients suspected of having quantitative abnormalities, eighteen bilateral and three unilateral biopsies from twenty-one fertile men with normal chromosome complements were investigated. A quantitative analysis was performed, employing the Sertoli cell number as a basis for reference. The inter-individual variation in the so-called 'Sertoli cell ratio' (germ cell/Sertoli cell) between individuals was relatively low and no significant difference between the right and left side was found.

It is concluded that the method has several advantages in studies using group controls.

INTRODUCTION

Analysis of the testicular biopsy has become an important tool in the study of testicular disorders in man. Testicular architecture has been defined in various diseases involving the gonads, such as Klinefelter's syndrome, hypogonadotrophic eunuchoidism and Sertoli-cell-only syndrome (Klinefelter, Reifenshtein & Albright, 1942; Nelson & Heller, 1945; Heller & Nelson, 1948; del Castillo, Trabucco & de la Balze, 1947). In several cases of testicular failure, however, the problem may be quantitative only, with a reduction in the number of one or more of the different types of germ cells (Paulsen, 1968). Such subjective observations of defects may be very difficult to standardize and even to detect by simple microscopy without quantitative analyses. Personal judgements may often be biased and it is difficult, or even impossible, to compare results from different laboratories. Even the same investigator may alter his criteria for normality. The need for more objective analyses of the seminiferous epithelium has been stressed by several investigators who have reported methods for quantitative analyses of the seminiferous epithelium (Roosen-Runge, 1956;

Mancini, Rosenberg, Cullen, Lavieri, Vilar, Bergada & Andrada, 1965; Steinberger & Tjioe, 1968; Rowley & Heller, 1971).

In the present and succeeding reports, data will be presented from quantitative analyses of human spermatogenesis. The series will comprise studies of: (a) infertile and fertile men with chromosome abnormalities, (b) men with severely reduced fertility and normal chromosome constitutions, and (c) fertile men with normal chromosome complements. This first report concerns the latter group.

MATERIALS AND METHODS

Bilateral testicular biopsies were obtained from twenty-one volunteers, aged from 19 to 39 years. To avoid having any individual with reproductive failure in the study, one to ten (usually three) semen specimens were examined before biopsy. Estimates of testicular size were obtained by using an orchidometer (a calibrated series of models of testes of different sizes). Sperm morphology analysis was performed by Dr R. Hammen (Hammen, 1944). Only candidates whose spermiograms showed an average sperm count of $>60 \times 10^6/\text{ml}$, $<50\%$ morphologically abnormal cells, and good motility were included in the investigation. All of the subjects were healthy and had normal reproductive histories. Normal secondary sex characteristics were found at physical examination. Simple routine blood and urine analyses were carried out before biopsy. All subjects had a normal 46,XY chromosome complement. Details of chromosome analyses and other relevant features of the individuals are given in Table 1.

Testicular biopsies were removed surgically under local anaesthesia, and were prepared according to the method of Rowley & Heller (1966). The biopsies were fixed in Cleland's fixative, embedded in Paraplast, sectioned serially at $4 \mu\text{m}$ and stained with iron-haematoxylin and eosin. Microscopic examination and quantification was performed by the first author with a Zeiss microscope using a $\times 40$ objective lens and $\times 12.5$ ocular lenses.

Randomly chosen cross sections of tubules were used for quantification (Rowley & Heller, 1971) after careful exclusion of areas with artefacts. Tangentially cut tubules with 'smeared' membranes and increased thickness of the germinal epithelium were avoided. A total of thirty tubular cross sections were counted. Usually thirty suitable tubules could not be found in one section of the biopsy. If additional sections were needed to complete the count, an interval of at least $40 \mu\text{m}$ was maintained between the sections to ensure that the same cells were not counted twice. The cells of the seminiferous epithelium were classified according to the criteria given by Rowley & Heller (1971) which are based on the work of Clermont (1963) and Heller & Clermont (1964).

Differentiation was made between types A dark (Ad), A pale (Ap) and B spermatogonia. Spermatocytes were classified as preleptotene (PL), leptotene (L), zygotene (Z) and pachytene (P) spermatocytes. Zygotene and pachytene spermatocytes were grouped together. Division figures from the two meiotic divisions were also recorded, as well as secondary spermatocytes. The relative frequency of these cell types is, however, very small and they have consequently been deleted from the tables. Part of the biopsy was prepared for a study of the

Table 1. Sperm analyses, chromosome counts and relevant features of twenty-one human subjects

Subject no.	Age	Marital status	Sperm analysis			Testis size (ml)	No. of children	No. of cells with various chromosome complements					
			No. of specimens	Sperm count $\times 10^6/\text{ml}$	Abnormal sperm heads (range of %)			Total no. of cells counted	Chromosome complement				
									<45	45	46	47	>47
1	19	Single	10	73	37 to 50	22	0	30			30		
2	40	Married	3	0*		22	3	30			30		
3	22	Single	3	174	23 to 28	22	0	30			29	1	
4	22	Single	3	291	22 to 22	25	0	30			29		1
5	37	Married	5	347	22 to 26	25	6	30		2	28		
6	26	Married	5	107	35†	23	0	30		1	29		
7	22	Married	4	230	26†	22	1	30			30		
8	26	Single	1	910	33†	20	4	30			30		
9	23	Divorced	3	101	39 to 45	18	2	15			14	1	
10	39	Divorced	3	358	48 to 51	28	8	15			15		
11	26	Married	3	192	40 to 49	25	2	15			15		
12	24	Divorced	3	99	28 to 34	22	0	15			15		
13	37	Married	3	84	21 to 34	32(R) 30(L)	3	15			14	1	
14	20	Married	3	43†	38 to 45	18	1	15		1	14		
15	29	Divorced	2	105	29 to 31	22	13	16		2	14		
16	31	Married	2	72	40 to 42	20	4	0§					
17	25	Divorced	2	147	31 to 35	20	4	15			15		
18	21	Married	2	71	38 to 54	25	1	15	1		13		1
19	28	Divorced	2	114	24‡	26(R) 23(L)	5	15			15		
20	23	Divorced	2	93	20 to 21	25	1	15		1	14		
21	31	Divorced	2	173	25 to 37	15	3	15		1	14		

R—right testis; L—left testis.

* Vasectomized.

† Counts may be low because they were taken only after biopsy (Rowley, O'Keefe & Heller, 1969).

‡ One specimen only.

§ The chromosome analysis was unsuccessful and an additional blood specimen could not be obtained.

Table 2. Sertoli cell ratios and total Sertoli cell number obtained from counts of thirty seminiferous tubule cross sections from twenty-one human subjects

Subject no.	Side	Spermatogonia				Spermatocytes			Spermatids		Sertoli cell no.
		Ad	Ap	B	Total	PL	L	Z—P	Sa—Sb	Sc—Sd	
1	R	0.75	0.89	0.31	1.95	0.25	0.16	1.91	2.35	1.73	352
	L	0.89	0.81	0.31	2.02	0.20	0.19	1.80	2.43	1.72	375
2	R	0.70	0.86	0.42	1.98	0.32	0.18	2.48	3.37	2.30	282
	L	0.75	0.85	0.41	2.00	0.23	0.29	2.74	4.81	3.35	255
3	R	0.54	0.68	0.27	1.48	0.25	0.21	1.91	3.19	2.30	254
	L	0.63	0.64	0.30	1.56	0.21	0.25	1.81	3.91	1.85	314
4	R	0.58	0.71	0.21	1.50	0.15	0.20	1.90	3.34	2.11	481
	L	0.47	0.62	0.22	1.31	0.18	0.20	2.18	3.39	2.18	330
5	R	1.07	1.28	0.48	2.83	0.33	0.32	2.50	4.87	3.63	211
	L	0.76	1.14	0.31	2.20	0.38	0.28	2.55	3.84	3.50	288
6	R	0.62	0.50	0.22	1.34	0.06	0.12	1.03	1.90	1.94	381
	L	0.53	0.62	0.20	1.35	0.16	0.12	1.06	2.32	1.59	426
7	R	0.53	0.61	0.19	1.33	0.15	0.12	1.56	2.78	1.49	377
	L	0.53	0.70	0.25	1.48	0.09	0.08	1.62	3.13	2.17	447
8	R	0.95	1.09	0.37	2.41	0.28	0.20	2.06	4.18	3.02	311
	L	1.08	1.11	0.29	2.48	0.22	0.31	1.92	3.68	2.40	315
9	R	0.68	0.78	0.21	1.67	0.20	0.23	1.72	2.48	1.45	421
	L	0.65	0.69	0.26	1.61	0.14	0.23	1.90	2.94	1.67	442
10	R	0.69	0.77	0.26	1.72	0.29	0.30	1.63	2.64	1.93	407
	L	0.66	0.79	0.21	1.66	0.40	0.21	1.80	2.35	2.01	373
11	R					Not successful					
	L	0.71	0.91	0.35	1.97	0.33	0.37	2.86	2.99	1.99	359

Table 2 (cont.)

Subject no.	Side	Spermatogonia				Spermatocytes			Spermatids		Sertoli cell no.
		Ad	Ap	B	Total	PL	L	Z—P	Sa—Sb	Sc—Sd	
12	R	0.51	0.61	0.23	1.35	0.25	0.25	1.75	2.67	2.10	567
	L	0.55	0.55	0.31	1.40	0.34	0.23	2.49	3.30	1.99	442
13	R	0.81	0.86	0.33	2.00	0.20	0.17	1.67	2.87	1.83	399
	L	0.91	1.00	0.27	2.19	0.26	0.32	1.72	2.28	1.69	336
14	R	0.80	0.72	0.30	1.83	0.44	0.38	2.33	3.60	2.48	287
	L	0.63	0.69	0.24	1.56	0.32	0.19	2.22	4.14	1.73	350
15	R	0.54	0.68	0.26	1.49	0.24	0.19	2.10	3.16	1.75	373
	L	0.60	0.88	0.29	1.77	0.22	0.24	2.15	2.74	2.13	339
16	R	1.09	1.03	0.25	2.37	0.34	0.20	1.95	3.13	2.30	367
	L	1.05	0.97	0.20	2.21	0.31	0.21	1.99	3.90	1.98	346
17	R	0.64	0.83	0.24	1.72	0.31	0.13	2.10	3.04	1.73	372
	L	0.67	0.78	0.16	1.61	0.21	0.21	2.10	2.65	1.66	389
18	R	0.59	0.80	0.19	1.58	0.25	0.21	1.44	1.59	1.53	421
	L					Not successful					
19	R	0.53	0.42	0.27	1.22	0.08	0.16	1.55	2.18	1.44	328
	L	0.42	0.41	0.21	1.05	0.24	0.22	1.35	1.94	1.75	393
20	R	0.63	0.98	0.24	1.85	0.37	0.25	2.31	3.49	3.45	289
	L	0.73	0.77	0.33	1.83	0.25	0.30	1.98	2.89	3.35	329
21	R					Not successful					
	L	0.86	1.01	0.36	2.23	0.23	0.26	2.42	2.64	2.19	357
Range		0.42 to 1.09	0.41 to 1.28	0.16 to 0.48	1.05 to 2.83	0.06 to 0.44	0.08 to 0.38	1.03 to 2.86	1.59 to 4.87	1.44 to 3.63	
Mean		0.70	0.79	0.28	1.77	0.25	0.22	1.96	3.05	2.14	361

For abbreviations, see text.

meiotic chromosomes, the results of which will be published separately (Skakkebak, Bryant & Philip, 1973).

Spermatids were classified as early spermatids, Sa and Sb, and late spermatids, Sc and Sd. In addition to the germ cells, all Sertoli cells with visible nucleoli were counted.

All germ cells that were cut through the geometric centre and which could fulfil the necessary criteria for identification, were included in the counts. The total number of counted germ cells of each of the different types was divided by the total number of Sertoli cells in the same cross sections of tubules. The resultant values are referred to as Sertoli cell ratios (SCR). The total number of each group of PL, L, Z + P, Sa + Sb and Sc + Sd were also related to the total number of spermatogonia.

TEXT-FIG. 1. Mean and range of Sertoli cell ratio in eighteen bilateral and three unilateral testicular biopsies taken from twenty-one human subjects. For abbreviations, see text.

RESULTS

The SCR from analyses of thirty-nine biopsies (eighteen bilateral and three unilateral) are presented in Table 2. The values for all spermatogonia were also grouped in order to use the results in forthcoming investigations on biopsies fixed in Stieve's fixative, since differentiation between types of spermatogonia is often difficult in such biopsies. The mean SCR of types Ad, Ap and B spermatogonia were 0.70, 0.79 and 0.28, respectively. The mean SCR of early spermatocytes, preleptotene and leptotene, were about equal, 0.25 and 0.22, respectively,

while the mean SCR of late spermatocytes, zygotene and pachytene, which were grouped together, was 1.96. In general, early spermatids were found in a greater number than late spermatids (mean SCR of the two groups, Sa+Sb and Sc+Sd, were 3.05 and 2.14, respectively). The SCR mean and ranges of the various groups of cells are illustrated in Text-fig. 1. These ranges are equivalent to estimates of the 2.5 and 97.5 percentiles of the frequency distribution for men of the category investigated (Reed, Henry & Mason, 1971). Means and ranges of the various spermatocyte/spermatogonia and spermatid/spermatogonia ratios are presented in Table 3.

Table 3. Spermatocyte: spermatogonia and spermatid: spermatogonia ratios for twenty-one human subjects

	<i>Spermatocyte : spermatogonia</i>			<i>Spermatid : spermatogonia</i>	
	<i>PL : S</i>	<i>L : S</i>	<i>(Z+P) : S</i>	<i>(Sa+Sb) : S</i>	<i>(Sc+Sd) : S</i>
Mean	0.14	0.13	1.13	1.76	1.23
Range	0.05 to 0.25	0.05 to 0.21	0.77 to 1.79	1.01 to 2.65	0.77 to 1.87

Means and range of values obtained by calculation on results from analysis of all thirty-nine testicular biopsies. For abbreviations, see text.

The following calculations were performed to test if the SCR for the right and left side of an individual could be assumed to be the same for any group of germ cells. For each case, where bilateral analysis was performed, the ratio between different groups of germ cells and Sertoli cells was determined in each of the thirty tubule cross sections from each side. The Mann-Whitney test was applied to the results from the two sides in each subject. No significant difference between the two sides was found (significance level: $P = 0.05$).

In order to obtain an estimate of the accuracy of the method, the following procedure was followed. The results from counts of thirty tubules in each of the thirty-nine biopsies were divided into six groups, each consisting of counts from five tubules. For each of the six groups the total number of (a) spermatogonia, (b) preleptotene+leptotene spermatocytes, (c) zygotene+pachytene spermatocytes, (d) Sa+Sb spermatids and (e) Sc+Sd spermatids were divided by the total number of Sertoli cells. Thus, the resulting values all represented the SCR for counts from five tubules. The standard deviation (s_i) for the SCR of each group of cells from an individual (i) was determined. The standard error of a mean of six SCRs (each based upon counts from five different tubules) was then calculated by the following formula:

$$SEM = \frac{s}{\sqrt{6}}, \text{ where } \bar{s} = \sqrt{\frac{1}{39} \sum s_i^2}$$

The standard error of the mean for Sertoli cell ratios of the various groups were: spermatogonia, 0.13; PL+L spermatocytes, 0.10; Z+P spermatocytes, 0.19; Sa+Sb spermatids, 0.36; Sc+Sd spermatids, 0.26. The standard errors of the means of six SCRs, all based on results from five different tubule sections

within an individual, may also be taken as estimates of the standard error of a mean of counts from thirty different tubules within an individual.

The variation between subjects is reflected in the various SCRs for a given cell type obtained from different subjects. In order to compare this inter-individual variation with the intra-individual variation, the F-ratio for a given cell type may be calculated as

$$F = \frac{s_{\bar{x}}^2 \text{ between individuals}}{s_{\bar{x}}^2 \text{ within individuals}}$$

The following F-ratios were found: spermatogonia, 9.6; PL+L spermatocytes, 2.0; Z+P spermatocytes, 5.0; Sa+Sb spermatids, 4.4; Sc+Sd spermatids, 5.6. As the 5%-significance limit for these F values is about 1.5, it can be concluded that the variation within a biopsy of an individual is smaller than the inter-individual variation.

DISCUSSION

The present study confirms that the variation of the SCR between individuals considered to have normal spermatogenesis is relatively small. Relative constancy of the SCR in the present study is important because one paramount aim of the investigation is to provide a reference for evaluation of spermatogenesis in patients suspected of having quantitative abnormalities. Although the method is objective, it is possible that the constancy of the SCR between subjects in the present investigation is due to the fact that the same person performed all counts. Larger differences between counts made by different persons may be due to discrepancies in distinguishing fragments and cells cut through the geometrical centre (in borderline cases, one person may include a given cell in the count while another person may regard it as a fragment and exclude it). The small difference in the average Sertoli cell number/tubule cross section between the investigation of Rowley & Heller (1971) (approximately ten) and the present study (approximately twelve) can be explained by such factors. Optimal amounts of information can probably, therefore, be drawn from studies where the same investigator has performed all the microscopic examinations. However, only minor variations seem to result from different persons performing counts, as reported by Rowley & Heller (1971). This fact is emphasized by the relatively small differences between the average SCRs reported by Rowley and Heller and those obtained in the present study.

Among the various methods for quantification of the cells of the seminiferous epithelium (Roosen-Runge, 1956; Mancini *et al.*, 1965; Steinberger & Tjioe, 1968; Rowley & Heller, 1971), the method using the Sertoli cell as a constant (Rowley & Heller, 1971) was chosen for several reasons. The use of a reference cell in the germinal epithelium itself compensates for possible changes in the testicular tissue such as shrinkage due to preparation, and changes in the tissue due to pathological or therapeutic circumstances. Animal studies have shown that Sertoli cells, unlike germ cells, are very resistant to changes caused by the influence of hormones or X-rays during experiments (Clermont & Morgentaler, 1955; Clermont & Perey, 1957; Oakberg, 1959; Lacy & Lofts, 1965; Heller,

O'Keefe & Heller, 1968). Divisions of Sertoli cells after sexual maturity have not been observed in laboratory animals nor in human individuals (Rowley & Heller, 1971; von Ebner, 1902; Nebel & Murphy, 1960; Roosen-Runge, 1962; Bloom & Fawcett, 1962; Steinberger & Steinberger, 1971).

This method is particularly useful for the measurement of alterations in the testes of subjects who are used as their own controls, since the SCR for a given cell type of a given individual at different times is smaller than the inter-individual variation (Rowley & Heller, 1971). Furthermore, the present study revealed that the variation within a biopsy of an individual is considerably smaller than the inter-individual variation, and the difference between right and left sides, as also demonstrated in the present study, is of no significance.

The method also has several advantages in studies using group controls. The Sertoli cell would provide a constant which would compensate for variation in shrinkage caused by different fixatives. Differences in tissue preparation techniques between laboratories are often unavoidable and in clinical studies of rare syndromes using material collected by different laboratories or by the same laboratory at different times, it is important that a standard be available. Since many of these patients never have a 'normal' testis to use as a control, a group control using the same constant factor, i.e. the Sertoli cell, offers the only objective comparative measurement of spermatogenesis that can be made.

The cell association method of Rowley & Heller (1971) was not considered for the present investigation since the primary aim was to provide a reference for studies on patients with various testicular disorders. In such cases, the organization of the seminiferous epithelium might well be lost and the cell association method be inapplicable.

The Sertoli cell cannot be used as a reference cell in tubules that are so severely damaged that the Sertoli cell itself is injured. This may be the case in the testes of sub-fertile males. Such testes, however, often reveal partly or totally damaged tubules together with well-preserved tubules. Valuable information on the composition of the seminiferous epithelium in the preserved tubules may be obtained in such cases. The method can also be used in cases with different types of tubules (Skakkebak, Hultén, Jacobsen & Mikkelsen, 1973), e.g. tubules with or without spermatogenic arrest. In such cases, information on tubules without obvious arrest could be provided and in tubules with subjectively abnormal spermatogenesis, a more objective description of the lesion could be obtained.

The various groups of germ cells were also related to the number of spermatogonia, since an inhibition of spermatogenesis at the spermatocyte or spermatid level should also be reflected by a decrease in these ratios. This may be of value in borderline cases having a small reduction in number of spermatocytes or spermatids, as indicated by the Sertoli cell ratio. If the spermatocyte/spermatogonia or the spermatid/spermatogonia ratio in such cases is also clearly abnormal, the assumption will be supported. In cases with a clear or suspected reduction in the number of spermatogonia, these ratios are naturally of no diagnostic value.

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