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Quantitation of the Cells of the Seminiferous Epithelium of the Human Testis Employing the Sertoli Cell as a Constant*

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Summary. Two variants of a method for determining the quantitative structure of the human seminiferous epithelium are described. Both versions (counting cells in whole tubules and in CA's) utilize specific criteria for the identification of cells, tubules and /or CA's. Both methods utilize the Sertoli cell as a constant with the cell types expressed as numbers of cells per Sertoli cell. One hundred biopsies were quantitated by the tubular method and 100 by the CA method. Counts were found to be reproducible and consistent. Comparisons were made between the two methods, different individuals doing the quantitation, quantitations of the right and left testes of the same subject and between biopsies from the same subject taken at different times. No difference was found between the right and left testes or biopsies from the same subject taken at different times. Quantitation of biopsies from one individual before and after testicular X-ray irradiation show how the method can be applied.

Key-Words: Seminiferous Epithelium — Human — Germinal cells — Testis—Male reproduction.

Introduction

Since von Ebner first attempted to classify the rat seminiferous epithelium in 1888, the structure and organization of the testis has been a matter of interest to both the investigator as well as the clinician. Until recently, human testicular histology has been less than definitive in allowing evaluation of normal versus abnormal conditions. Clermont (1963) and Heller and Clermont (1964) described the cycle of the human seminiferous epithelium and its organization into cell associations. Thus a basis was established for the development of a definitive quantitative method for evaluating differences between testes as well as testicular changes occurring in a single testis.

We herewith report a method by which one is able to quantitate the cells of the human germinal epithelium using the Sertoli cell as a constant. The method may be applied in two ways: (1) counting all identifiable cells in sections of whole tubules; or (2) counting the cells present in distinct cell associations only. It can be used to compare testicular function before and after treatment or to compare normal and abnormal testes.

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3001458

Materials and Methods

The approaches to the quantitation method (tubule and cell association) are identical in many respects. The general procedure including tissue methods, cell descriptions and the statistics of the quantitation is in section A. Section B deals with the cell association (CA) approach and section C with the tubular approach.

A. General Procedure

1. Tissue Methods. Testicular biopsy specimens were obtained from normal male volunteers (age range 21 to 52 years). Reproductive normalcy was determined from history and physical examination as well as by hormonal studies, sperm counts and testicular biopsy examination. Specimens were fixed in Cleland's fixative and at least 100 4 μ serial sections were stained with iron hematoxylin and eosin (Rowley and Heller, 1966). All counting was performed using either the ×40 dry or ×100 oil immersion objective lens and the ×12.5 eyepieces. Counting was performed on coded slides and by the same person and/or different persons.

2. Cell Descriptions and CA Descriptions. The following brief diagnostic description of the individual cells applies to tissue fixed in Cleland's solution and stained with iron hematoxylin and eosin. For a description of human germinal cells fixed in Zenker's solution and stained with PAS or Harris hematoxylin and eosin see Heller and Clermont (1964).

The A dark (Ad) spermatogonium has an oval nucleus with fine dark almost homogeneous chromatin. Often a large vacuole is found in the nucleus and one or more nucleoli may occur along the nuclear membrane or at the edge of the vacuole. The cell adheres closely to the tubular wall. The cytoplasm appears clear and unstained. The A pale (Ap) spermatogonium also has an oval nucleus and lies along the tubular wall. The nuclear chromatin is fine and pale staining with the appearance of ground glass. The thin nuclear membrane has one or two nucleoli attached to it and the cytoplasm is clear. The B spermatogonium has a round nucleus containing a centrally placed nucleolus coated with clumps of chromatin. The membrane of the nucleus is also lightly covered with flakes of chromatin although the nuclear material is of the same ground glass appearance as the Ap. Although serial sections reveal that this cell is always touching the tubular wall, it may sometimes appear to be above the Ad or Ap and the attachment may not be apparent.

The preleptotene spermatocyte (resting or R) resembles the B spermatogonium and these two cells can appear alike. The R has a round nucleus and is usually above the tubular wall. The nucleolus is centrally located and is more heavily covered with chromatin than the nucleolus of the B spermatogonium. Large clumps of chromatin resembling small nucleoli may also be found either on the nuclear membrane or within the nucleoplasm. The remaining nucleoplasm is darker and filled with chromatin granules. The nucleus of the leptotene spermatocyte (L) is finely filamentous. The filaments may be very short, hardly more than spicules, or they may be longer, but are arranged in no definite order. This cell is found above the spermatogonia and is not attached to the basement membrane. The nucleus of the zygotene spermatocyte (Z) is distinctive in that the long thin chromosome pairs form a "bouquet" arrangement apparently "knotted" close to the nuclear membrane. The pachytene spermatocyte (P) nucleus enlarges in size and the chromosomes contract to become short and thick. A large, round, well-defined nucleolus appears during the first half of its 15-day existence. Secondary spermatocytes (SS) resemble Sa spermatids, except for their distinctly larger size. Multiple dark chromatin masses are apparent against the light homogeneous chromatin of the nucleoplasm.

The Sa spermatid nucleus has pale slightly granular chromatin containing a few flakes of dark chromatin. The nucleus is always centrally located within the cytoplasm. The Sb spermatid is characterized by a denser, smaller nucleus which is slightly asymmetrical in shape. During its development into an Sb_2 the nucleus changes becoming "hatchet" shaped with the larger end touching the cell membrane on the side facing the tubular wall. It also becomes dark staining and homogeneous with some nuclear condensation apparent as small darker areas. Although a caudal tube and flagellum form during this time it is difficult to see them. The Sc spermatid has a greatly condensed nucleus with the tip pointing toward the tubule wall. The long narrow channel of cytoplasm, extending from the back of the head toward the tubule lumen, resembles the wake of a motorboat. The flagellum and caudal rings are visible. The Sd spermatid flattens

to resemble the head of an arrow v
view. The early Sd is embedded de
lumenal surface of the tubule. The c
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The Sertoli cell nucleus is larg
lumenal side of the nuclear membra
is irregularly shaped. The cytopla
are not visualized in these preparat

There are six CA's made up of
characterizing each of the six CA's

CA	Spermatog	
I	Ad	Ap
II	Ad	Ap
III	Ad	Ap
IV	Ad	Ap
V	Ad	Ap
VI	Ad	Ap

A tubule cut in cross section ma
cells forming the boundaries of two

3. Counting. During quantitatio
fashion similar to performing blood
observed until a statistically signi
section is used for counting if one se
number of tubules or CA's. The edge
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Both longitudinal and cross sec
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For both the tubule and CA appro
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B. Cel

1. Each recognizable complete C
2. The type of division figures see
3. A total of 30 CA's completes a
4. Care is taken to avoid areas of

The main prerequisite for the use
by the person performing the scoring.
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tures somewhat during the duration o
the late portion of a CA as compared t
For instance, an Sb spermatid chang

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to resemble the head of an arrow with the base of the head thicker than the point from a side view. The early Sd is embedded deeply in Sertoli cytoplasm and the late Sd is situated on the lumenal surface of the tubule. The cytoplasm is discarded from the flagellar area and is visible as the residual body (Rb) when the mature cell leaves the Sertoli cell cytoplasm.

The Sertoli cell nucleus is large and pale staining with at least one deep infolding in the lumenal side of the nuclear membrane. The large round nucleolus is covered with chromatin and is irregularly shaped. The cytoplasm is extended between the germinal cells but its borders are not visualized in these preparations.

There are six CA's made up of groupings of these cells. The composition of cellular types characterizing each of the six CA's is as follows:

CA	Spermatogonia			Spermato-cytes		Spermatids	
I	Ad	Ap	B	—	P	Sa	Sd
II	Ad	Ap	B	—	P	Sa	Sd
III	Ad	Ap	—	R	P	Sb	—
IV	Ad	Ap	—	L	P	Sb	—
V	Ad	Ap	B	L	P	Sc	—
VI	Ad	Ap	B	Z	Dia.	Sc	—

A tubule cut in cross section may contain more than one cell association (Fig. 1) and the cells forming the boundaries of two adjoining CA's are usually mixed.

3. Counting. During quantitation of the germinal cells testicular sections are scanned in a fashion similar to performing blood smear differential counts. The biopsy section is carefully observed until a statistically significant sample is obtained. Approximately every tenth section is used for counting if one section is not sufficient to obtain a statistically significant number of tubules or CA's. The edges of the section and any parts of the section showing artifacts such as tissue tearing or great amounts of blood in the interstitium are avoided.

Both longitudinal and cross sections of tubules with clear lumina are used for scoring (Fig. 2). All cells are counted only when cut through their geometrical center (center of nucleus or nucleolus). Cells are quantitated only in tubular areas where the thickness of the germinal epithelium is minimal and constant. The obviously "tangentially" cut tubules or parts of tubules are avoided in quantitation. Therefore areas eliminated are those with: a. More than one layer of spermatogonia (Ad and Ap), b. Wide basement membrane (it appears "smeared" away from the tubule and has no distinct outer edge), and c. Increased thickness of the germinal epithelium (the distance from basement membrane to lumen).

For both the tubule and CA approaches, 30 counted areas are needed to achieve a statistical validity of $P = 0.05$. After 30 areas are counted the numbers of cells are totaled. To obtain the Sertoli cell ratio, the total of each cell type is divided by the total number of Sertoli cells. For instance, if the total number of Ad's equals 60 and the total number of Sertoli cells is 100, the ratio for Ad's would be 0.6 ($60/100 = 0.6$).

B. Cell Association Approach

1. Each recognizable complete CA is counted.
2. The type of division figures seen (meiotic or mitotic) are noted.
3. A total of 30 CA's completes a normal count.
4. Care is taken to avoid areas of mixed CA's.

The main prerequisite for the use of this approach is complete ease in identifying the CA's by the person performing the scoring. Emphasis must be placed on the cellular changes that occur during each CA. The cells must not be considered as being static in form. Each cell matures somewhat during the duration of each CA. A cell may appear to be slightly different in the late portion of a CA as compared to the same cell type in the early portion of the same CA. For instance, an Sb spermatid changes considerably during CA III. In order to quantitate

3001460

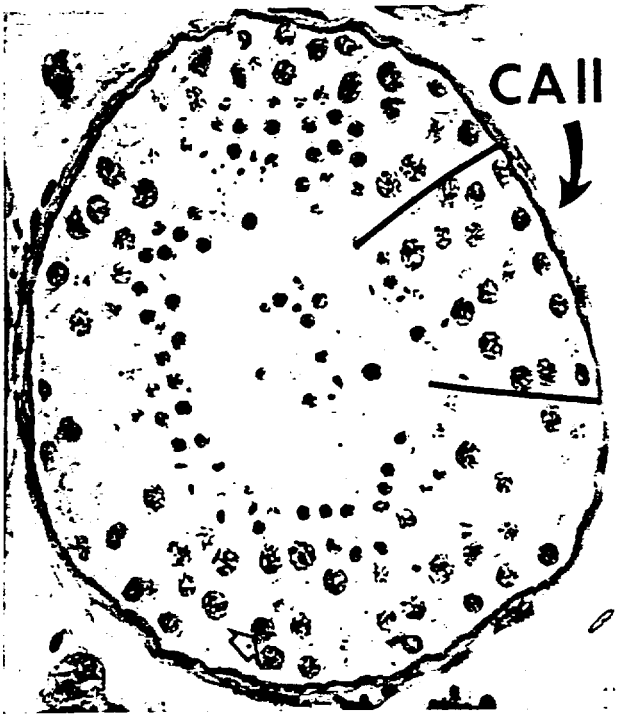

Fig. 1. Photomicrograph of a seminiferous tubule from a normal man. This entire tubule would be counted if a tubular count were being done. In a CA count only the area between the lines (*CA II*) would be counted. Open arrow points to a Sertoli cell that is easily identifiable and yet would not be counted because no nucleolus is visible. Cells free in the lumen would not be counted. ×320

correctly, the individual doing the counting must know the nature of the maturation changes that occur for each cell type in each CA. In other words, the individual doing the quantitation must be able to identify each cell exactly, whether it is associated with the other cells of the correct CA or not. This will prevent the counting of areas containing mixed CA's. Excluding A dark, A pale, and B spermatogonia, a CA is considered adequate for quantitation only when all other cell types are present. The Ad and Ap spermatogonia exist in each CA and are counted when encountered, but are not required for making the CA identification.

Cell association VI provides an exception because of its very short duration and because of the many cells encountered due to the two meiotic divisions. Thus each of the cell types that comprise this CA are rarely present concurrently. Cell association VI is counted if most of the cell types are present. Examples of cell combinations in CA VI which would be counted are:

Ad, B, Diakinesis, Div. fig., SS, Se
Ad, Ap, Z, P, Div. fig., Se
Ap, B, Z, Div. fig., Se
Ad, Ap, B, Z, SS, Se

C. Tubular Approach

1. Whole tubular cross sections and side areas of longitudinally cut tubules are used if they fit the general requirements.
2. Tubules with clear lumina are used whenever possible.

Fig. 2. Photomicrograph of a norm
sectioned tubule and round tubul
suitable CA's in these areas could
tubul

3. Every cell that meets the g
cell mixing.
4. The only areas eliminated f
lumen or without sharply defined b
5. Thirty tubules or areas compl
counted in biopsies having germinal
Although this method is simple
must be especially careful in identi
fragments of cells which can be easil
to avoid counting fragments is to c
cells of the same type. Unless it is ab
or larger diameter than surrounding

To determine the constancy
calculated the average number o
for each of 25 biopsies. This w
number of Sertoli cells per tubu
the counts of 750 tubules. It wa

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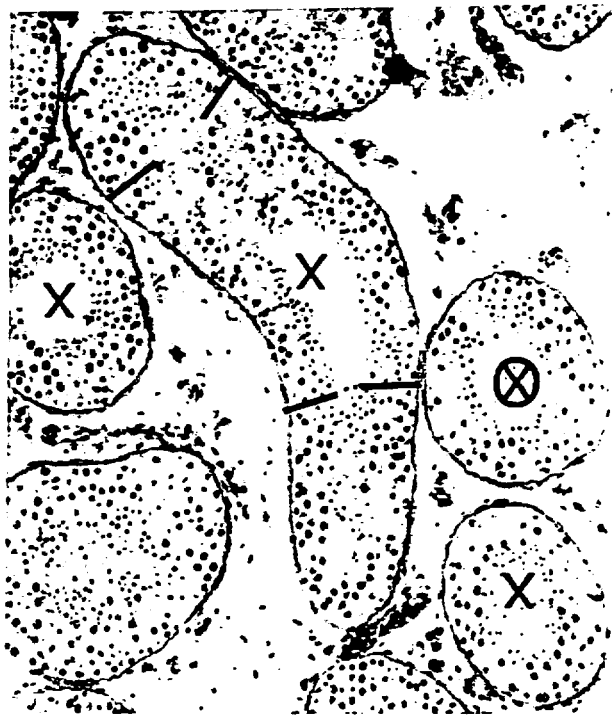

Fig. 2. Photomicrograph of a normal human testis. Areas between lines in the longitudinally sectioned tubule and round tubules with X in them can be used for tubular counts. Any suitable CA's in these areas could also be used for a CA count. Tubule with ⊗ is the same tubule enlarged in Fig. 1. ×100

3. Every cell that meets the general requirements is counted without regard to CA or cell mixing.

4. The only areas eliminated from the count are tubules containing artifacts, without lumen or without sharply defined basement membranes, or any tangentially cut area.

5. Thirty tubules or areas complete a count of a well populated biopsy. Sixty tubules are counted in biopsies having germinal cell depletion.

Although this method is simpler since the CA's need not be identified, the investigator must be especially careful in identifying the cells .Care should be taken to avoid counting fragments of cells which can be easily identified (such as pachytene spermatocytes). One way to avoid counting fragments is to compare the size of the nucleus with that of surrounding cells of the same type. Unless it is abnormal a cell will not have a nucleus with a much smaller or larger diameter than surrounding cells in the same stage of development.

Results

To determine the constancy of Sertoli cell numbers in the normal testes we calculated the average number of Sertoli cells per 30 round cross sections of tubules for each of 25 biopsies. This was found to be 299.0 ± S.E. 10.62. The average number of Sertoli cells per tubule was determined separately and calculated from the counts of 750 tubules. It was found to be 10.0 ± S.E 0.3.

3001462

Table 1. *Evaluation of 100 normal biopsies counted by the cell association method and the tubule method; averages of Sertoli cell ratios and standard errors for each cell*

	Ad	Ap	B	R	L	Z	P	Sa	Sb	Sc	Sd
					Cell association method						
Ave.	0.65	0.60	0.31	0.24	0.57	0.08	2.46	1.63	1.63	1.59	2.03
S.E. ±	0.02	0.02	0.02	0.01	0.03	0.01	0.09	0.05	0.09	0.08	0.09
					Tubule Method						
Ave.	0.64	0.59	0.24	0.16	0.25	0.04	1.98	1.60	1.01	1.00	1.03
S. E. ±	0.02	0.02	0.01	0.01	0.01	0.01	0.06	0.06	0.04	0.03	0.03

One hundred normal testicular biopsies were counted by the CA method and 100 normal biopsies were counted by the tubular method. The Sertoli cell ratios and standard errors for each method are shown in Table 1.

The Sertoli cell ratios and standard errors found in the CA method reflect the remarkable constancy of cell counts between biopsies of different individuals. It can also be seen that for every ten Ad's tabulated approximately ten Ap's, five B's, four R's, ten L's, one Z, 30 or 40 P's, and 30 or 40 each of Sa, Sb, Sc, and Sd spermatids will also be tabulated. For every ten Ad's tallied, about 15 Sertoli cells will be found.

The Sertoli cell ratios and standard errors resulting from the tubular method reflect even better consistency between biopsies probably due to the larger numbers of cells counted. Slight differences are apparent, however. For every ten Ad's counted, ten Ap's, four or five B's, four R's, five L's, one Z, 30 or 40 P's, and 15 to 30 each of Sa, Sb, Sc, and Sd spermatids will be tabulated by this method. About 15 Sertoli cells will be tabulated for every ten Ad's.

In each method the Ad and Ap spermatogonia were statistically equal in numbers. The B spermatogonia and the R spermatocyte ratios were lower than the Ad and Ap in each method. The pachytene spermatocyte count was the same for both methods. In two instances, very marked differences were noted. The ratios of the leptotene spermatocytes and the late spermatids were significantly lower using the tubular method for counting. In the CA method approximately the same ratio for each of the four spermatid types was maintained. In contrast, the ratios of the four spermatid types were lower for the later spermatids using the tubular count method, probably due to sloughing into the lumen.

The individual ratio for each cell type was always higher using the CA method than in the tubular count method, albeit the differences were not statistically significant for most cell types (Table 1).

Comparison between several individuals quantitating by both methods on the same biopsy is shown in Table 2. Comparisons were also made between right and left testis of the same subject (Table 3) and between biopsy specimens from the same subject obtained at different times (Table 4). It was concluded that neither counting performed by different individuals nor counting the cells of the same subject taken at different times or from right and left testis varied significantly.

Table 2. *Comparison of results ob*
single biopsy using first the cell a

Individuals performing the quantitation		Cell type		
		Ad	Ap	B
CA	B	0.65	0.71	0.
	A	0.56	0.52	0.
	C	0.55	0.57	0.
	D	0.63	0.45	0.
TUB	D	0.55	0.59	0.
	A	0.61	0.52	0.
	B	0.60	0.57	0.
	C	0.61	0.51	0.

[a] The same individuals are de

Table 3. *Comparison of results be*

Individuals performing the quantitation	Side	Cell type	
		Ad	Ap
Biopsy No. 1			
A	Right	0.56	0.52
A	Left	0.57	0.46
Biopsy No. 2			
B	Right	0.60	0.48
B	Left	0.51	0.52

Table 4. *The variation in quantitati*
in counts from biopsies taken 18 mo.

Individual performing the quantitation	Cell type		
	Ad	Ap	B
Biopsy No. 1			
B	0.53	0.61	0.23
Biopsy No. 2			
B	0.52	0.56	0.21

To test the usefulness of the
cular biopsies were quantitate
X-ray irradiation and recorde

3001463

Table 2. *Comparison of results obtained by four individuals*[a] *performing the quantitation on a single biopsy using first the cell association (CA) method and then the tubular method (TUB)*

Individuals performing the quantitation		Cell type										
		Ad	Ap	B	R	L	Z	P	Sa	Sb	Sc	Sd
CA	B	0.65	0.71	0.31	0.23	0.85	0.08	2.77	1.75	1.53	1.52	2.33
	A	0.56	0.52	0.16	0.16	0.61	0.19	2.04	1.36	1.78	1.31	1.86
	C	0.55	0.57	0.17	0.21	0.61	0.12	1.60	1.76	1.40	1.22	1.57
	D	0.63	0.45	0.17	0.24	0.87	0.16	2.78	1.51	1.60	1.18	1.54
TUB	D	0.55	0.59	0.33	0.12	0.25	0.01	1.91	1.93	1.16	1.08	0.93
	A	0.61	0.52	0.15	0.10	0.23	0.01	1.61	1.31	0.84	0.94	0.82
	B	0.60	0.57	0.21	0.21	0.33	0.02	1.60	1.11	1.07	1.02	0.83
	C	0.61	0.51	0.24	0.10	0.18	0.05	2.63	2.07	0.87	0.95	1.16

[a] The same individuals are designated as A, B, C, and D throughout.

Table 3. *Comparison of results between right and left testis of two subjects using the tubular method*

Individuals performing the quantitation	Side	Cell type										
		Ad	Ap	B	R	L	Z	P	Sa	Sb	Sc	Sd
Biopsy No. 1												
A	Right	0.56	0.52	0.16	0.06	0.31	0.09	2.04	1.26	1.88	1.21	0.86
A	Left	0.57	0.46	0.23	0.06	0.26	0.07	2.76	2.44	1.84	1.83	1.53
Biopsy No. 2												
B	Right	0.60	0.48	0.19	0.20	0.32	0.02	1.76	1.39	1.02	1.16	0.99
B	Left	0.51	0.52	0.27	0.17	0.34	0.05	2.15	1.19	1.50	1.01	1.09

Table 4. *The variation in quantitation between biopsy specimens from the same subject is shown in counts from biopsies taken 18 months apart from the same individual on the same testis using the tubular method*

Individual performing the quantitation	Cell type										
	Ad	Ap	B	R	L	Z	P	Sa	Sb	Sc	Sd
Biopsy No. 1											
B	0.53	0.61	0.23	0.14	0.35	0.05	2.50	1.07	1.78	0.99	1.28
Biopsy No. 2											
B	0.52	0.56	0.21	0.23	0.33	0.03	2.46	1.29	1.70	1.22	1.27

To test the usefulness of the tubular method, the germinal cells in serial testicular biopsies were quantitated before and after testicular exposure of 200 r of X-ray irradiation and recorded in Table 5. This clearly reveals which cells were

3001464

Table 5. *Sertoli cell ratios of biopsies taken from one subject before and after 200 r of testicular X-ray irradiation. All biopsies were quantitated by one individual by the tubular method*

Time after irradiation	Cell type											No. Sertoli cells
	Ad	Ap	B	R	L	Z	P	Sa	Sb	Sc	Sd	
Control	0.60	0.48	0.19	0.20	0.32	0.02	1.76	1.39	1.02	1.17	0.99	242
22 hours	0.67	0.53	0.08	0.15	0.12	0.07	1.87	1.18	1.06	1.00	1.27	269
14 days	0.52	0.29	0.07	0.01	0.02	0	1.20	0.89	1.21	0.95	0.74	236
29 days	0.22	0.17	0.02	0	0.004	0	0.004	0.39	0.41	0.54	0.65	284
84 days	0.04	0.02	0	0	0	0	0.002	0	0.005	0.10		408
151 days	0.02	0.01	0.002	0	0	0	0	0	0	0	0	464
232 days	0.07	0.05	0.005	0	0	0	0.005	0	0	0	0	440
399 days	0.46	0.21	0.10	0.01	0	0	0.22	0.09		0.05		320
568 days	0.63	0.50	0.25	0.13	0.24	0.001	1.46	1.30	1.28	1.00	1.01	280

most radio-sensitive and to what degree. In addition, the degree of tubular shrinkage was revealed by the increasing number of Sertoli cells per 30 tubules from normal of 242 to 464. The rate of cellular recovery can also be followed.

Discussion

Attempts have been made to quantitate the human germinal epithelium using Chalkley's technique (Roosen-Runge, 1956; Roosen-Runge, Marberger and Nelson, 1957), using tubule cross sections as a constant (Mancini *et al.*, 1965) and using "units" of tubular wall as a constant (Tjioe *et al.*, 1967; Steinberger and Tjioe, 1968). Chalkley's technique is not applicable to the measurement of relative numbers of cells since it measures only the relative area of all cells (Eschenbrenner *et al.*, 1948). The second method (utilizing cross sections of tubules as a constant) is not applicable for quantitatively measuring changes because the tubules themselves change and therefore, do not represent a constant factor. The third method (using unit length of tubule wall as a constant), is inapplicable as well because it does not compensate for tubular shrinkage. Steinberger and Tjioe (1968) demonstrated the degree of variability revealed when fixed point counting is not used for Sertoli cells.

Methods for the quantitation of the germinal epithelium using the Sertoli cell as a constant have been developed and used extensively for the mouse (Oakberg, 1959) and rat (Clermont and Morgentaler, 1955; Clermont and Harvey, 1967; Clermont and Perey, 1957).

The Sertoli Cell as Used in Quantitation

It is known that the mass of the testis (i.e., the interstitial area and the seminiferous tubules) does not always remain constant during alteration of its function. Therefore, any method measuring changes in cell numbers must introduce a factor compensating for this change. The quantitative procedures described above for the germinal cells use the Sertoli cell as a reference cell or constant compensating

factor and are expressed as a r
Sertoli cells.

The Sertoli cell is used be
adult animals and its resista
stability has been demonstrat
and Clermont and Perey (1957
treatments as irradiation (O
hormones by hypophysectomy
and Lofts, 1965) and FSH (He

The Sertoli cell lends itsel
does not undergo mitotic divi
Runge, 1962; von Ebner, 190
and Fawcett, 1962). The san
division in man by the followir
tion of several thousand radi
normal men) containing tritia
despite the abundant presence
submitting to administration
gonadotropin also failed to rev
have studied biopsies obtained
or having a variety of clinical
division of a Sertoli cell.

The Sertoli cell is used to
manner. All tissues are cut at
the total mass of the testis bec
both in length and width. The
portion of the total Sertoli cel
testis. For example, a control
tubules counted. A biopsy of th
citrate contained 371 Sertoli c
216 A dark spermatogonia wer
biopsy for 30 tubules. If interp
appears to have increased in
relating the A dark cell to the
for the control and 0.60 for the
Therefore in biopsies taken wh
(or normal and abnormal) adul
cells may be used as a constant

The use of the Sertoli cell
treatment of the infantile tes
unknown whether the therapy
cell cannot be used as a consta
or injury such as testicular infe
cells themselves are affected.

Counting cells by the CA
been of particular importance

3001465

factor and are expressed as a ratio of the number of the cell type to the number of Sertoli cells.

The Sertoli cell is used because of its known stability of number in normal adult animals and its resistance to change during alterations of the testis. Its stability has been demonstrated by studies of Clermont and Morgentaler (1955) and Clermont and Perey (1957). Its resistance to change has been found after such treatments as irradiation (Oakberg, 1959), removal of influences of pituitary hormones by hypophysectomy (Clermont and Morgentaler, 1955), estrogen (Lacy and Lofts, 1965) and FSH (Heller *et al.*, 1968).

The Sertoli cell lends itself particularly well to use as a constant because it does not undergo mitotic division following sexual maturity in animals (Roosen-Runge, 1962; von Ebner, 1902; Nebel and Murphy, 1960; Greep, 1966; Bloom and Fawcett, 1962). The same conclusion was reached for its lack of mitotic division in man by the following observations in our laboratory. Careful examination of several thousand radioautographed sections of testicular material (from normal men) containing tritiated thymidine revealed no labeling of Sertoli cells despite the abundant presence of normal labeling of germinal cells. Normal men submitting to administration of X-ray irradiation, Nilevar or human chorionic gonadotropin also failed to reveal labeled Sertoli cells. Over a period of years we have studied biopsies obtained from approximately 2000 adult men either normal or having a variety of clinical problems without having identified a single mitotic division of a Sertoli cell.

The Sertoli cell is used to compensate for change in mass in the following manner. All tissues are cut at 4 μ thickness. Following a debilitating treatment the total mass of the testis becomes less and the seminiferous tubules may shrink both in length and width. Then the same 4 μ thickness will contain a greater proportion of the total Sertoli cells (and germinal cells and interstitial area) of the testis. For example, a control biopsy contained 304 Sertoli cells in the 30 round tubules counted. A biopsy of the same individual after treatment with clomiphene citrate contained 371 Sertoli cells in 30 round tubules. In the same two biopsies, 216 A dark spermatogonia were counted in the control and 261 in the treatment biopsy for 30 tubules. If interpreted by counts of the A dark cell alone the A dark appears to have increased in number. However, correcting for shrinkage by relating the A dark cell to the Sertoli cells of the same tubules gives ratios of 0.62 for the control and 0.60 for the treatment. This reveals that no change occurred. Therefore in biopsies taken where a comparison between a treated and untreated (or normal and abnormal) adult male is to be made, we conclude that the Sertoli cells may be used as a constant when quantitating any cell in the testis.

The use of the Sertoli cell as a constant may not apply during stimulatory treatment of the infantile testes of hypogonadotropic eunuchoids. It remains unknown whether the therapy would stimulate Sertoli cell division. The Sertoli cell cannot be used as a constant in testes that are severely damaged by disease or injury such as testicular infections or Klinefelter's syndrome where the Sertoli cells themselves are affected.

General Discussion

Counting cells by the CA method as opposed to the tubular method has been of particular importance in helping to understand the kinetics of nor-

3001466

mal human germinal epithelium. The precision and reproducibility of a count done within the stringent guidelines of this form of method has revealed unexpected information considering the relatively small number of cells actually counted (around 1000 cells per count). The precision is revealed in several ways. For instance, while studying the results of the 100 control counts, it was discovered that in any one count of 30 CA's about seven CA I, five CA II, five CA III, four CA IV, seven CA V, and two CA VI could be expected. There were never more CA II or CA III than CA I or CA V. Although, occasionally, a count was completed without a CA VI, there were never more than three in any one quantitative effort. In further examination, the percentage of the total number of cells in any CA was found to be very uniform. Upon the assumption that the duration of each CA is directly proportional to the number of cells occupying it at any one time, the duration of each CA and of each cell type was able to be recalculated (Heller *et al.*, 1969) using the greater number of subjects involved here.

Intriguing relationships have been emphasized by the CA counts. The distribution of mitotic figures seen in each CA was found to confirm that shown by Clermont (1966a) i.e., a peak only in CA II. This peak probably represents the division of type B spermatogonia into preleptotene spermatocytes. This raises some serious questions as to the time of division of as well as the duration of the Ad or Ap spermatogonia. If Clermont's supposition that Ad and Ap divide only during CA V is correct, a very large peak should also be found in CA V. However, each CA appears to contain about equal numbers of mitotic divisions in proportion to its duration (excluding CA II). This suggests in turn that Ad and Ap spermatogonia are undergoing division at any and all times during the cycle of the germinal epithelium. In turn this signifies that the Ad or Ap may not have a precise beginning or end. As a result their duration cannot be calculated at this time.

The CA method reveals that "perfect" CA's are encountered only infrequently as compared to the total tubular area. It would seem that if all of the germinal cells are part of some perfect CA, more perfect CA's than mixed areas would be found. An explanation for the problems of random mitotic figures and large areas of mixed CA's can be made. If random division of a spermatogonium is accepted, then a new CA would be started by that division. For instance, if an Ad spermatogonium divided in CA III, the CA relationship would remain intact until the Ap began division into type B spermatogonia. Immediately the CA's would be mixed, as B's would be found when the more mature cells are in CA IV (B's are not acceptable in CA IV). As the B's mature and divide into preleptotene spermatocytes, two CA's would be occupying the same tubular area, one above the other. Eventually, the lower cycling group would replace the upper one and a "pure" CA would again be found. Of course the first erratic division need not begin with the Ad spermatogonium. In fact, it would probably start with whatever cell is the stem cell (A_S). We already know that at least one other more primitive spermatogonial type (AL), does exist in the human (Rowley *et al.*, 1971) from observations using the electron microscope. Since this cell is not yet easily identified in paraffin sections or readily distinguished from Ad, it has probably been included in the Ad assessment.

As noted, the CA method resulted in slightly higher numbers of R, L, Z, P, Sa, Sb, Sc, and Sd cells than the tubular method. Since many CA's are incomplete

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3001467

they were omitted in the CA method. A CA by definition, includes each of the stated cells, whereas in the tubular method incomplete CA's and mixed areas are included. Thus this difference could be predicated beforehand. The incomplete CA's are in all likelihood due to either attrition by cell death, erratic spermatogonial division or in the case of spermatids, attrition by premature sloughing into the tubular lumen.

The relationship of any one cell type to any other cell type is dependent upon whether a division occurred between the two cell types and the duration of existence of each cell. Therefore, we were able to determine the ratios of cell types to each other by dividing the Sertoli cell ratio of any cell type by its duration for each cell and using the resultant figure of the less differentiated cell as 1. Since it is known that each spermatocyte forms four spermatids, the theoretically expected ratio for R:L:Z:P:Sa:Sb:Sc:Sd should be 1:1:1:1:4:4:4:4. The actual ratio determined from CA counts was 1:1:1:1+:3:4:4:4 and from tubular counts was 1:1:$^1/_2$:2:4:4:3:3. (These figures cannot be directly compared with those obtained by Clermont (1966b) for spermatogonia, as the methods used for counting were very different.) The slightly lower numbers of spermatids found in the tubular counts are probably due to attrition by premature sloughing. The larger numbers of pachytenes in the tubular counts are probably due to the extreme ease with which this cell is identified. The pachytene is also the largest cell and possibly less care was used unconsciously in eliminating fragments of these cells thereby giving an overcount. This is less likely to happen in a CA count because of the emphasis on counting criteria.

The relationship of spermatogonia to spermatocytes and spermatids was not determined because the duration of these cells has only been estimated. The type B spermatogonia by definition divides at the end of CA II, when it forms preleptotene spermatocytes, however, its exact beginning sometime during CA V is not known. Therefore even the duration of type B is inexact (9.5 days$\pm$2) and cannot be used to establish this ratio.

The tubular method of quantitation, although perhaps inferior for use in delineating basic normal testicular cell relationships, is ideal to compare the cells before, during and after testicular alteration. This is so because the CA's are usually not left intact after a debilitating or traumatic effect has taken place. A decision should be made before the quantitation is performed as to what use is to be made of the results in order to make the appropriate choice of methods which then should be continued throughout the experimental evaluation.

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3001468

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