A METHOD FOR THE QUANTIFICATION OF LEYDIG CELLS IN MAN

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Summary. The use of the Leydig cell/Sertoli cell (LC/SC) ratio as a representative index for the number of Leydig cells present in the testis provides an objective method for their quantification. It reflects changes in the number of Leydig cells that might occur as their activity is depressed or stimulated by various agents. The Sertoli cell number in the adult male does not appear to be altered by administration of drugs, hormones or irradiation in dosages typically used in clinical investigations of germinal and Leydig cells. Thus, the Sertoli cell may be utilized as a constant.

Thirty-eight testicular biopsies were taken from nineteen normal men. Quantification of the biopsies, using the LC/SC ratio method revealed that: (1) no statistically significant variation could be found in biopsies taken from the same testis of the same subject at different times; (2) biopsy ratios between subjects ranged from 0.19 to 0.72; and (3) a statistical difference in ratios between right and left testis of the same subject occurred in four out of eleven subjects.

It is concluded that the LC/SC ratio is an accurate and reproducible quantitative measure for comparing a given subject against his own control when a further biopsy is taken from the same testis.

INTRODUCTION

Leydig cell function in man is known to be readily stimulated or readily depressed by the administration of hormonal agents (Maddock & Nelson, 1952; Maddock, Epstein & Nelson, 1952; Leach, Maddock, Tokuyama, Paulsen & Nelson, 1956; Heller, Laidlaw, Harvey & Nelson, 1958; Heller, Moore, Paulsen, Nelson & Laidlaw, 1959; Tamm, Apostolakis & Voight, 1965; Cleveland, Ahmad, Sandberg & Savard, 1966; Heller, Lalli & Rowley, 1966; Bardin, Ross & Lipsett, 1967; Heller, Rowley & Heller, 1969), chemicals (Johnson, 1969) and irradiation (Heller, Wootton, Rowley, Lalli & Brunca, 1965). With these changes in function, a change in number may or may not

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occur. Any conclusions in the literature of changes in Leydig cell numbers were drawn subjectively. At present, there is no objective method which will provide a satisfactory quantification of the possible changes. One of the obstacles to overcome in developing a method is that almost any agent which may alter Leydig cell function or numbers also changes seminiferous tubular diameter and length and thus overall testicular volume. An accompanying alteration in interstitial cell concentration follows. This may lead to the subjective impression that Leydig cell numbers have been altered.

For determining Leydig cell numbers, a point of reference is required that may be used as an index for making comparisons in the face of changes in testicular volume resulting from possible alterations in interstitial volume and seminiferous tubules.

Of several potential constants, the numbers of Leydig cells per tubule, per unit area, per blood capillary, and per number of Sertoli cells were investigated. Only the Sertoli cell proved suitable. None of the other parameters proved consistent or reproducible from biopsy to biopsy in the same man. Each was found to be unsatisfactory because, in every case, the reference chosen as a constant did not remain so and changed with alteration of testicular function. However, there is substantial evidence that during typical clinical or biological investigation, the use of drugs, hormones or irradiation to alter reproductive function does not alter the number of Sertoli cells (Clermont & Morgentaler, 1955; Clermont & Perey, 1957; Oakberg, 1959; Nebel & Murphy, 1960; Lacy & Lofts, 1965; Greep, 1966; Clermont & Harvey, 1967; Heller, O’Keefe & Heller, 1968).

**MATERIALS AND METHODS**

Nineteen normal men, ranging in age from 24 to 47 years, volunteered as subjects for repeated testicular biopsies. The normality of their reproductive system was confirmed on the basis of physical examinations, sperm counts (twenty weekly collections/subject), urinary testosterone and germinal cell cytology. Thirty-eight testicular biopsies were obtained by the method of Rowley & Heller (1966). The biopsies were fixed in Cleland’s solution to produce good nuclear detail. One hundred and fifty to 200 serial 4-μm sections were cut from each biopsy specimen and stained with iron haematoxylin and eosin.

In order to define the area or areas of the histological section to be counted, low magnification photographs were made of the areas selected for use as a guide during scoring at higher magnification. These photographs served as a guide to define the perimeters of the areas to be scored.

The sample areas were photographed on 35-mm film at a total magnification of ×25. The area of the biopsy reproduced on the film was 1.33 × 0.9 mm. To achieve greater contrast, a green filter was employed. Photographs were taken using the following precautions:

1. to avoid artifacts, edges of the biopsy sections were not photographed;
2. to ensure a good sampling of the biopsy and to avoid the possibility of
counting the same cells twice, the area to be photographed was at least 40 \( \mu m \) (ten sections) away from the next area;

(3) to avoid sampling one area of the biopsy exclusively, different areas within the sections were photographed.

Care was taken in making the 3 in. \( \times \) 4 in. prints at a total magnification of \( \times 75 \) to include all edges of the negative and to make certain that all nucleoli on the edges of the photographs were in focus. Each Sertoli cell and each Leydig cell having a distinct nucleolus lying wholly or in part within the sample area of the biopsy was counted using the microscope at the higher magnification of \( \times 400 \).

A correction factor to reconcile the differences in total Leydig cell and Sertoli cell volume was unnecessary since only cells sectioned through their nucleoli were counted. Since the nucleoli of Leydig and Sertoli cells are approximately the same size, the chance of encountering a section through one cell type more often than another was negligible.

In general, counting ten histological sections yielded a Leydig cell/Sertoli cell (LC/SC) ratio which was within 10% of the true ratio with probability \( P = 0.90 \). Fifteen to twenty different photographs were made initially from which ten were selected at random for quantification. If the ratio obtained from these ten was not satisfactory, additional photographs were immediately available for completion of the quantification.

The LC/SC ratio for the entire biopsy was obtained by taking the sum of all Leydig cells and Sertoli cells counted from the different sections. The variance, standard deviation and standard error were computed from the individual LC/SC ratios of the different sample areas. More than ten pictures had to be assessed if the coefficient of variation of the sample was higher than 20%. The number of pictures needed, \( N \), was estimated by the formula:

\[
N = \frac{(1.64)^2 \sigma^2}{0.01 \mu m^2}
\]

where the standard deviation of the sample was used for \( \sigma \) and the LC/SC ratio of the sample was used for \( \mu m \). To meet these statistical requirements, the biopsy must be of adequate size. All results were analysed using Student's \( t \) test.

RESULTS
To determine the usefulness and reproducibility of the method, three variables were evaluated.

(a) Comparison of biopsies from the same subject taken at different times

The number of Leydig cells from biopsies taken on the same testis of the same subject at differing times did not vary significantly \( (P = 0.05) \). Table 1 reveals that the results of quantifying the same testis were constant even when three biopsies were compared and when biopsies were obtained as long as 57 months apart.

(b) Comparison of biopsies between subjects

The comparison of Leydig-cell numbers between the nineteen subjects revealed a range in the LC/SC ratio of 0.19 to 0.72 with a mean of 0.39 (Table 2).
Table 1
Comparison of the Variability in Quantification of the Same Testis in the Same Subject at Different Times

<table>
<thead>
<tr>
<th>Subject no.</th>
<th>Date of biopsy</th>
<th>Testis side</th>
<th>Leydig cell/Sertoli cell ratio ± S.E.</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>3.8.67</td>
<td>Right</td>
<td>805/3050 = 0.26 ± 0.03</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>21.9.67</td>
<td>Right</td>
<td>670/2448 = 0.27 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>44</td>
<td>16.6.66</td>
<td>Right</td>
<td>1956/3698 = 0.53 ± 0.03</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>3.8.67</td>
<td>Right</td>
<td>1374/2516 = 0.55 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>180</td>
<td>10.1.63</td>
<td>Right</td>
<td>2049/3996 = 0.51 ± 0.03</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>21.9.67</td>
<td>Right</td>
<td>1681/3229 = 0.52 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>206</td>
<td>26.1.67</td>
<td>Right</td>
<td>1102/3216 = 0.34 ± 0.03</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>3.8.67</td>
<td>Right</td>
<td>850/3081 = 0.28 ± 0.03</td>
<td></td>
</tr>
<tr>
<td></td>
<td>21.9.67</td>
<td>Right</td>
<td>1415/4141 = 0.34 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>206</td>
<td>26.1.67</td>
<td>Left</td>
<td>1084/3064 = 0.35 ± 0.03</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>3.8.67</td>
<td>Left</td>
<td>1165/3297 = 0.33 ± 0.03</td>
<td></td>
</tr>
<tr>
<td></td>
<td>21.9.67</td>
<td>Left</td>
<td>1364/3687 = 0.37 ± 0.03</td>
<td></td>
</tr>
</tbody>
</table>

N.S., Not significant.

* For this report, \( P < 0.05 \) is the criterion for statistical significance.

Table 2
Comparison of the Variability Between Subjects

<table>
<thead>
<tr>
<th>Subject no.</th>
<th>Testis side</th>
<th>Leydig cell/Sertoli cell ratio ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>227</td>
<td>Right</td>
<td>2700/5934 = 0.46 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>Left</td>
<td>3083/4510 = 0.68 ± 0.03</td>
</tr>
<tr>
<td>180</td>
<td>Right</td>
<td>2049/3996 = 0.51 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>Right</td>
<td>1681/3229 = 0.52 ± 0.04</td>
</tr>
<tr>
<td>262</td>
<td>Right</td>
<td>876/4277 = 0.20 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Left</td>
<td>1286/5896 = 0.22 ± 0.01</td>
</tr>
<tr>
<td>224</td>
<td>Left</td>
<td>2220/5660 = 0.39 ± 0.02</td>
</tr>
<tr>
<td>223</td>
<td>Right</td>
<td>2955/5521 = 0.54 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>Left</td>
<td>2794/7995 = 0.35 ± 0.02</td>
</tr>
<tr>
<td>189</td>
<td>Right</td>
<td>3534/6162 = 0.57 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Left</td>
<td>3843/6769 = 0.57 ± 0.02</td>
</tr>
<tr>
<td>204</td>
<td>Right</td>
<td>2994/8016 = 0.37 ± 0.02</td>
</tr>
<tr>
<td>30</td>
<td>Right</td>
<td>670/2448 = 0.27 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>Right</td>
<td>805/3050 = 0.26 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>Left</td>
<td>769/2634 = 0.29 ± 0.02</td>
</tr>
<tr>
<td>219</td>
<td>Left</td>
<td>1074/3323 = 0.32 ± 0.03</td>
</tr>
<tr>
<td>218</td>
<td>Right</td>
<td>1056/3956 = 0.27 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Left</td>
<td>1111/5845 = 0.19 ± 0.01</td>
</tr>
<tr>
<td>170</td>
<td>Right</td>
<td>1882/8996 = 0.21 ± 0.01</td>
</tr>
<tr>
<td>232</td>
<td>Right</td>
<td>3173/8507 = 0.37 ± 0.01</td>
</tr>
<tr>
<td>260</td>
<td>Right</td>
<td>1848/6751 = 0.27 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Left</td>
<td>1122/4410 = 0.25 ± 0.02</td>
</tr>
<tr>
<td>44</td>
<td>Right</td>
<td>1956/3698 = 0.53 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>Right</td>
<td>1374/2516 = 0.55 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>Left</td>
<td>3076/4614 = 0.67 ± 0.03</td>
</tr>
<tr>
<td>206</td>
<td>Right</td>
<td>1102/3216 = 0.34 ± 0.03</td>
</tr>
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<td></td>
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<td>1165/3297 = 0.35 ± 0.03</td>
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<td></td>
<td>Left</td>
<td>1084/3064 = 0.33 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>Left</td>
<td>1364/3687 = 0.37 ± 0.03</td>
</tr>
<tr>
<td>199</td>
<td>Right</td>
<td>1528/3365 = 0.45 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>Left</td>
<td>2852/6109 = 0.47 ± 0.02</td>
</tr>
<tr>
<td>251</td>
<td>Left</td>
<td>5067/7037 = 0.72 ± 0.04</td>
</tr>
<tr>
<td>243</td>
<td>Right</td>
<td>3059/7117 = 0.43 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>Left</td>
<td>4165/8683 = 0.48 ± 0.02</td>
</tr>
<tr>
<td>138</td>
<td>Left</td>
<td>2064/9227 = 0.22 ± 0.01</td>
</tr>
</tbody>
</table>
(c) **Comparison of biopsies from the right and left testes of the same subject**

The comparison of LC/SC ratios between right and left testis of the same subject obtained at the same time revealed a significant difference in four of the eleven men. The ratios of the right and left testis did not vary significantly in seven men ($P = 0.05$) (Table 3). Statistical analysis showed that the variation between subjects was greater than between right and left testes of the same subject. Reproducibility was revealed by re-counting the same biopsy by the same person and by different persons. The variation in counts performed by any two investigators averaged less than 6% and the variation in counts by the same investigator was within 3%.

<table>
<thead>
<tr>
<th>Subject no.</th>
<th>Right testis</th>
<th>Left testis</th>
<th>$P$*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Leydig cell/Sertoli cell ratio ± S.E.</td>
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<td></td>
</tr>
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<td>227</td>
<td>2700/5934 = 0.46 ± 0.03</td>
<td>3083/4510 = 0.68 ± 0.03</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>262</td>
<td>876/4277 = 0.20 ± 0.01</td>
<td>1286/5896 = 0.22 ± 0.01</td>
<td>N.S.</td>
</tr>
<tr>
<td>223</td>
<td>2953/5521 = 0.54 ± 0.03</td>
<td>2794/7995 = 0.35 ± 0.02</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>189</td>
<td>3534/6162 = 0.57 ± 0.01</td>
<td>3843/6769 = 0.57 ± 0.02</td>
<td>N.S.</td>
</tr>
<tr>
<td>30</td>
<td>805/3030 = 0.26 ± 0.03</td>
<td>769/2634 = 0.29 ± 0.02</td>
<td>N.S.</td>
</tr>
<tr>
<td>218</td>
<td>1056/3956 = 0.27 ± 0.01</td>
<td>1111/5845 = 0.19 ± 0.01</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>260</td>
<td>1848/6751 = 0.27 ± 0.01</td>
<td>1122/4410 = 0.25 ± 0.02</td>
<td>N.S.</td>
</tr>
<tr>
<td>206</td>
<td>1102/3216 = 0.34 ± 0.03</td>
<td>1165/3297 = 0.35 ± 0.03</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>850/3081 = 0.28 ± 0.03</td>
<td>1084/3064 = 0.35 ± 0.03</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>1415/4141 = 0.34 ± 0.03</td>
<td>1364/3687 = 0.37 ± 0.03</td>
<td>N.S.</td>
</tr>
<tr>
<td>44</td>
<td>1956/3698 = 0.53 ± 0.03</td>
<td>3076/4614 = 0.67 ± 0.03</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>199</td>
<td>1528/3365 = 0.45 ± 0.03</td>
<td>2852/6109 = 0.47 ± 0.02</td>
<td>N.S.</td>
</tr>
<tr>
<td>243</td>
<td>3059/7117 = 0.43 ± 0.02</td>
<td>4169/8683 = 0.48 ± 0.02</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

N.S., Not significant.

* For this report, $P < 0.05$ is the criterion for statistical significance.

**DISCUSSION**

The LC/SC ratios reveal that the number of Leydig cells remains remarkably constant for the same testis in a given subject over a period of time. Hence, analysing biopsies of the same testis in the same individual before and following an investigative procedure should reveal changes with a validity of $P = 0.05$. Conversely, since the two testes of the same individual have approximately a 40% chance of being statistically dissimilar, comparing the right testis used as a control with the left testis after treatment could be misleading.

Similarly, using the average LC/SC ratio of a group of normal controls for assessing the results of a given procedure upon an individual or a small group of individuals could be even more misleading. Therefore, each subject must serve as his own control and each testis must also serve as its own control.

To date, no correlation has been forthcoming to rationalize the great
differences in LC/SC ratios between different subjects. Age was found not to be a factor as no significant correlation \((r = -0.23)\) was found between age and the LC/SC ratios of the subjects. Other possibilities to be considered are that, indeed, some individuals may have more Leydig cells than others, or, conversely, some may have more Sertoli cells than others. Although the total number of Sertoli cells per subject may vary, further stability of the Sertoli cell was demonstrated by counting the number of Sertoli cells per tubular cross section. The average number of Sertoli cells per tubule for all subjects studied was 10.03 \(\pm\) 0.3. Table 4 reveals that the number of Sertoli cells/tubular cross section for the two subjects whose LC/SC ratios lay at the extreme ends of the range averaged 10.36 \(\pm\) 0.6, well within the average range found for all subjects.

### Table 4

**Comparison between Sertoli Cells/Tubular Cross Section for Two Subjects Whose Leydig Cell/Sertoli Cell Ratios Lay at Extreme Ends of the Range**

<table>
<thead>
<tr>
<th>Subject no.</th>
<th>Testis side</th>
<th>Leydig cell/Sertoli cell ratio (\pm) S.E.</th>
<th>Sertoli cell/tubular cross section (\pm) S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>218</td>
<td>Right</td>
<td>0.27 (\pm) 0.01</td>
<td>879/83 = 10.59 (\pm) 0.42</td>
</tr>
<tr>
<td></td>
<td>Left</td>
<td>0.19 (\pm) 0.01</td>
<td>1004/90 = 11.16 (\pm) 0.47</td>
</tr>
<tr>
<td>251</td>
<td>Right</td>
<td>0.75 (\pm) 0.04</td>
<td>676/90 = 8.5 (\pm) 0.38</td>
</tr>
<tr>
<td></td>
<td>Left</td>
<td>0.72 (\pm) 0.04</td>
<td>894/80 = 11.18 (\pm) 0.48</td>
</tr>
</tbody>
</table>

It is conceivable that a higher testosterone production may be correlated with either a change in Leydig-cell numbers or a change in Leydig-cell size. The increased testosterone production with human chorionic gonadotrophin is correlated with an increase in their size (Heller & Leach, 1971), whereas following irradiation, preliminary analysis in our laboratory reveals that subsequent diminution in testosterone secretion may be correlated to an increase in Leydig cell numbers, thereby suggesting a compensatory mechanism.

Other methods for quantifying Leydig cells were unsatisfactory because control biopsies could not be compared with biopsies following administration of drugs and hormones. The method of Chalkley (1943) determines the volume of Leydig cells present in a sample, but does not reveal numbers.

Sargent & McDonald (1948) related the number of Leydig cells to the number of tubules present on any given area of the biopsy. However, this did not provide for changes in the size of the tubules following administration of various agents. Clegg (1961), using rats, modified Sargent & McDonald’s method by deriving a mathematical formula to compensate for shrinkage of the tubules. A uniform distribution of the cells in the testes was assumed along with a similar shrinkage of the tubules with regard to diameter and length. Since there is no evidence that a similar ratio of shrinkage between diameter and length of tubules occurs in man, we could not use this method.

The method of Dykes (1969) measures the relative proportions of tubules to areas of Leydig cells in normal and pathological testes. However, this point counting method makes no mention of Leydig-cell numbers, only of area and volume, and does not take into account the possible changes in tubular size.
which may occur following administration of various agents to the normal subject. Ahmad, Lennox & Mack (1969) tried to improve on Dykes' method by measuring the total volume of the testes and the proportion of the testes occupied by the cells. This method could not be used because it also makes no mention of Leydig-cell numbers. The authors did not have an adequate series of normal controls since the testicular material was obtained post mortem and no definite conclusions were reached because of the small number of cases studied.

The fact that the Sertoli cells are located in the tubules and the Leydig cells in the interstitial area does not enter into the statistical analysis. This is because the tubules are not found in one area of the testes and the interstitial tissue in another. Since the tubules and Leydig cells are homogeneously mixed throughout the entire testes, the LC/SC ratio is assumed to be a normally distributed random variable. In species where the tubules and interstitial tissue are in separate discrete areas, this assumption is not valid and, therefore, the method cannot be used to quantify Leydig cells.

The biopsy specimens are obtained from a limited area of the dorsal side of the testes immediately below the tunica albuginea to avoid any disturbance of the epididymis. Only a small area of the testis is sampled, not the entire organ, so the homogeneity of the biopsy specimens and of the association of cells to tubules is stable because the area of the testis sampled is always the same.

The LC/SC ratio is valid as long as a statistically random sample is scored. In testing the method, the authors have counted an excess of twenty sample areas of the biopsy specimen. The investigator may quantify a larger number of sample areas of the biopsy to obtain the LC/SC ratio, though it is only necessary to count a minimum of ten and statistically evaluate the data as set forth in the method. If the data are not statistically valid, more areas may be counted, though the LC/SC ratio from counting ten sample areas is usually within 10% of the true ratio (P < 0.90).

When dealing with human tissue, one is limited to the use of biopsies, except in some pathological cases. Because it is impossible to weight the entire organ and study it as such, this method enables the investigator to obtain information concerning the relative number of Leydig and Sertoli cells in any individual man. Since it utilizes the least amount of tissue with the least harm to the individual, the investigator will be able to determine the effects of any investigational procedure on the same individual by means of multiple biopsies over a period of time.

ACKNOWLEDGMENTS

We wish to thank Florence Teshima for her excellent assistance in preparing the biopsy material used in the studies. We thank Dr Kathleen B. O'Keefe for her statistical evaluation of the data.

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