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Stereological methods as efficient and unbiased tools to quantitate structures in the testis

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Key terms Leydig cells, Sertoli cells, stereology.

During the last few decades the effects of different environmental agents on the testis have been investigated in animal experiments. Changes in the numbers or volume of the different cell types in the testis have been widely used end points in these studies. However, careful analyses of the methods used in quantitative studies are needed before the conclusions of these investigations can be accepted. Generally speaking, quantification in the testis has been carried out by profile counting in semithin sections, measurement of relative areas, or ratios of the cell type studied. Most of the methods used are known to result in biased estimates (eg, because the number of profiles counted in the sections depend on the size and shape of the structure counted (see figure 1) (1). Based on assumptions about the shape and size of the structures, various mathematical correction formulas have been applied to correct for these biases in conventional profile counting designs (1, 2).

Stereological methods

During the past few decades new stereological tools have been introduced (3). One of the new methods, optical fractionation, which is based on a systematic uniform sampling scheme, a fractionator design, and a 3-dimensional counting probe, the optical dissector, has proved to be a highly efficient stereological method with which to estimate the total number of cells in an organ, such as the testis (3, 4). The principal advantages of stereological methods include (i) the uniformity of the sampling, which ensures that all objects in the testis have the same probability of being sampled [eg, all cells in the testis have one and the same chance to be sampled (figure 1)] and (ii) the lack of assumptions needed about the shape, size, or orientation of the cells or shrinkage of the organ during histological processing.

Human testes. For information about the total numbers of Leydig and Sertoli cells in human testes, the numbers of these cells were estimated in 20 testes from men brought to the Department of Forensic Medicine, Copenhagen, due to sudden unexpected death.

A known fraction of the tissue was sampled systematically at random from each testis in a careful stepwise sampling procedure. Each testis was cut into 4-mm thick slabs (providing 8—12 slabs). Every 2nd or 3rd slab was sampled and cut into 4-mm thick bars providing 6—10 bars. Every 2nd or 3rd bar was sampled and cut into cubes. Every 4th to 6th of the cubes (approximately

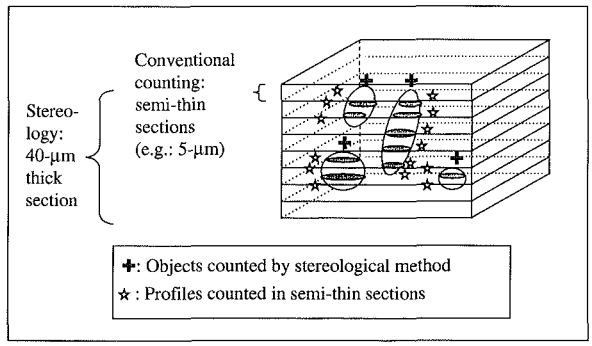

Figure 1. Bias of a conventional profile counting of objects in histological sections. Fourteen profiles are counted if profiles are counted in a conventional way in all semithin sections. For example, all the particles in this example have been counted more than once, and the large structures have been counted with a higher probability than the small structures. Four objects are counted if the director principle is applied. There are 4 particles in a tissue block.

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8—10 cubes) was sampled. The sampled tissue was embedded in 2-hydroxy-methacrylate (Technovit 7100®) and stained with hematoxylin eosin, a stain in which the Leydig and Sertoli cells were easily recognized. The blocks of methacrylate, each containing 8–10 cubes of testicular tissue, were cut into 40-μm thick sections. Approximately 10 sections were sampled from each testis, and the optical dissector principle was used to count the number of Sertoli and Leydig cells in a known fraction of each section (figure 2). By this sampling procedure the coefficient of variation (standard deviation/mean) at each sampling level was kept below 10%. The volume of the dissectors was chosen so that approximately 150 cells of each cell type were counted in each testis. The total numbers of Sertoli and Leydig cells were estimated by multiplying the counted number of cells by the inverse of the sampled fraction (eg, in 1 testis every 2nd slab, every 3rd bar, every 6th cube, and every 12th methacrylate section were sampled, and the Leydig cells were counted in 1/2000 of the tissue section. The estimated number of Leydig cells was equal to $2 \times 3 \times 6 \times 12 \times 2000 \times$ the counted number of Leydig cells.)

The mean total number of Sertoli cells in the testes from a normal man was estimated to be approximately 900×10^6 [coefficient of variation (CV) = 0.24, coefficient of error (CE) = standard error of the mean (SEM)/mean = 0.05). The mean total number of Leydig cells was estimated to be approximately 150×10^6 (CV 0.41, CE 0.09).

Animal experiments. The same principles were used to estimate the total number of Leydig and Sertoli cells in 10 rat testes.

The design was the same as that used for humans, except for some adjustments in the sampling procedure.

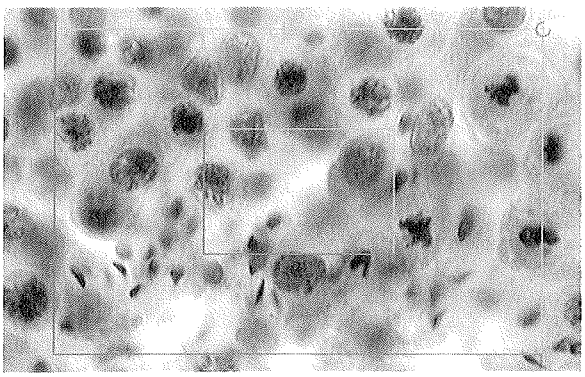

Figure 2. Section for which counting was done in the optical dissector in the 3-dimensional space by stepping through the thick tissue sections. The cells are counted when they are in focus and then counted only once. Sertoli cells are counted in the small frame and Leydig cells in the larger one. The size of the counting frame is decided from the estimation of the coefficient of variation due to this step in the sampling procedure.

The mean total number of Sertoli cells in the testes from rats was estimated to be approximately 20×10^6 (CV 0.08, CE 0.02). The mean total number of Leydig cells was estimated to be 19×10^6 (CV 0.12, CE 0.04). Thus a rat testis contains 11×10^6 Sertoli cells and 13×10^6 Leydig cells per gram of testicular tissue, while human testes possess 24×10^6 Sertoli cells and 5×10^6 Leydig cells per gram.

General considerations about quantitative studies of the testis. In the study of the human testis the total Leydig and Sertoli cell numbers, estimated by use of the new stereological methods, differed to some degree from the results obtained from previous studies. The estimated total Sertoli cell numbers have been reported to range from 390 to 3700×10^6 and the total Leydig cell number from 400 to 800×10^6, calculated from 2-dimensional profile counting (5—8). These large ranges can be ascribed to the difficulties in interpreting the results obtained from the assumption-based designs described earlier in detail (9, 10). Animal studies comparing quantification by assumption-based methods with stereological methods without assumptions about shape or shrinkage of the quantitated structures have shown that conventional counting methods may bias the results in unpredictable ways (eg, the results were influenced by different shrinkage of the tissue in hormone-treated rats and control rats) (9,10). Thus only unbiased methods should be used in quantitative investigations of the testis.

Applying these methods in the study of testes requires combination with staining techniques and functional markers. As an example, the optical dissector requires thick histological sections, which have practical implications to, for example, the staining techniques. However, we have shown that, with conventional staining techniques, staining in 40–μm thick sections and therefore counting in optical dissectors is possible for testis.

Perspectives in relation to toxicology

In light of the general decline in male reproductive health, monitoring of the number of Sertoli cells, which is essential for normal spermatogenesis, would give important new information. In addition the detection of possible changes in the number of germ cells over time could provide significant new knowledge on the monitoring of male reproductive health. An efficient and relatively quick method like the optical fractionator principle would be ideal in such monitoring. We have described the estimation of numbers as an example of stereology, but other parameters, such as the mean cell volume and surface area of cells or the length of the structures, can also be quantitated stereologically (3). The principles have also been applied to frozen sections and to electron microscopy, and thus the stereological techniques are applicable to all experimental toxicologic studies in which the

quantification of compartments of the testis, of cells, or of organelles are wanted.

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