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STEREOLOGICAL SAMPLING PRINCIPLES AND METHODS FOR THE MORPHOMETRY OF ARTICULAR CARTILAGE

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ABSTRACT

The application of stereological methods to morphometry of the articular cartilage is considered. The lateral tibial condyle of the knee joint of eight rabbits was examined. The thickness of the articular cartilage was measured, and, as stereological ratio estimators, the volume density and the numerical density of cells was estimated by point counting. The significance of random sampling for validity and precision of stereological estimators is discussed.

INTRODUCTION

A firm theoretical basis (Miles, 1978; Miles and Davy, 1976, 1977) with practical applications (Cruz-Orive and Weibel, 1981) has resulted from the recent development of stereology. Much attention has been paid to statistical aspects, i.e. to validity and precision of estimators.

The application of this model-free, design-based theory of unbiased estimation to the morphometry of articular cartilage poses some practical difficulties. Problems arise especially when sampling the 3-d specimen.

SAMPLING

The randomization of section plane, T, within the specimen, X, is of utmost importance for the validity of

stereological ratio estimators. The systematic sample can be considered as one random sample of complex form. The randomness of sections provides isotropic orientation and uniform distribution (IUR, Miles and Davy, 1976) of T in X. Two different relationships exist between X and T (Miles, 1978): (1) the 'restricted' case, in which T, provided that it is considered as a plane probe of constant size, is big enough to intersect X totally (unbounded probe), and (2) the 'extended' case, in which T is very small in relation to X.

In stereology, some subdomain Y (object phase of interest) of X is measured. We write $Y \subset X$. The typical ratio estimator is of form:

$$R = k(\alpha(Y \cap T)/A(X \cap T)) \qquad (1)$$

where $\cap$ means 'intersection between', α stands either for A (=area) or $(4/\pi)B=(4/\pi)$ times boundary trace length or some other fundamental stereological formula, and k is the corresponding coefficient for 3-d derivations. In the restricted case, in which usually both the numerator and the denominator of the ratio are random variables, setting T at random orientation and then intersecting X at random position results in biased estimators on account of the covariance between $\alpha(Y \cap T)$ and $A(X \cap T)$ (Miles and Davy, 1976). It has been shown that the ratio estimators can be made unbiased by 'weighting' the distribution of IUR planes T in X by the value of $A(X \cap T)$ (AWR-sampling; Miles and Davy; 1976). This is done by locating X in the xyz-coordinate system, generating random xyz-points, and, when the point hits X, by taking randomly orientated section at this point. In the extended case, in which X can be considered to be composed of small sub-x_i-units (T intersects x_i rather than X), unbiased ratio estimators can be achieved by taking x_i by IUR and then by taking arbitrary section from X_i (no weighting in section taking is needed). In practise, the AWR-sampling of X in the extended case can be carried out by cutting X into many small blocks, x_i, of equal size and by picking up a number of them at random. One arbitrary section per block is cut.

In the morphometry of articular cartilage the basic parameter is the thickness of the cartilage, which denotes the amount of cartilage tissue. The measurement of the thickness from sections provides that sections are obtained perpendicularly to the articular surface down to the subchondral bone. On the other hand, the size and the shape

of cells and the organization of the cartilage tissue varies in different distances from the articular surface. When this structural gradient is to be studied, the section must, of course, include constituents of it. These reasons cause that the section angle has to be fixed, which in turn means that neither isotropic nor AWR-planes can be obtained in the restricted case and this leads to biased estimators. In the extended case, the unbiasness of stereological ratio estimators depends on the relationship between the geometry of the specimen of interest and the section angle. It is to be noted, however, that the estimation of the volume density is independent of the section angle (Weibel, 1979).

Which one of the sampling cases is encountered in the case of articular cartilage, the restricted or the extended one? As a specimen we used the lateral tibial condyle of the knee joint of eight rabbits. Articular surfaces of condyles were sliced by a Leitz 1600 saw microtome into rectangles of equal size (0.3 x 1.5 mm: the shape was chosen on account of orientation of the blocks for thickness measurements, one 1 µm section was cut from the 0.3 mm side of the block). Cartilage was dissected free from the subchondral bone. Altogether 30-40 blocks (with cartilage thickness ranging between 0.2 mm and 0.8 mm) per condyle were obtained. A number of blocks was picked up at random. Therefore, considering the dimensions of the cartilage of the lateral tibial condyle of the rabbit, the articular cartilage can be regarded as 'extended' along with its surface but 'restricted' in direction of its thickness. As a consequence, possibilities for AWR-sampling existed. Because blocks with varying volumes had equal probability to be picked up by simple random sampling, a bias was introduced. However, the sample was uniformly random. At the same time the 'edge effect' (Weibel, 1979) of articular surface was negligible. The design-bias caused by the variation of cartilage thickness is of order 1/n, where n is number of sections.

Blocks were treated and processed for microscopical analysis by the method of Shepard and Mitchell (1977). One 1 µm thick epon section per block was obtained and stained with iron hematoxylin and Safranin O. The Holmes effect (Weibel, 1979) was effectively diminished when thin epon sections were examined.

The second sampling level was the field sampling or subsampling of section by using test systems for stereological estimation. Various sampling methods at this level

are discussed thoroughly by Miles and Davy (1977), Cruz-Orive and Weibel (1981), and Jensen and Gundersen (1982). We decided to divide the whole thickness of uncalcified articular cartilage, on account of its structural gradient, into three strata. In each stratum, h, the fields were put systematically into two adjacent rows perpendicularly to the articular surface. The location of the first field in each stratum was determined according to the principle of fixed orientation uniform random sampling (FUR, Miles and Davy, 1977). The field had to locate by its width completely inside the section. The thickness of the superficial stratum was selected constant, and the remaining thickness of the cartilage was halved into strata II and III.

ESTIMATION

The thickness of the articular cartilage was measured by a scale ruler from the image produced by the Wild Heerbrugg microscope at three systematically chosen points in each section at the final magnification of x280. By using mean thickness of the cartilage in the section the animal and population means were computed. Subsequently, variances of the estimators were computed. The total observed variance in the whole population was estimated as well as the variance within one animal (=between sections). The variance between the animals was achieved by subtracting the variance of the mean, SEM^2, within one animal from the total variance. The precision of the estimators was given as the relative SEM (RSE). They were computed by dividing SEM by the mean.

For stereological estimations, a simple square lattice was used as the test system. The lattice contained 7x7 points; d=1.4 cm at a magnification of x1400 (for point density in test systems, see Mathieu et al., 1981 and Weibel, 1979). The point density of those points (R_1) hitting cells (y_1), and the density of nuclear profiles (R_2), were counted (the number of nuclear profiles within a test area = y_2). Because the denominator of a ratio estimator in stratum I was constant, means and variances could be computed as usual. The thickness of strata II and III were random variables and the following estimators had to be used:

$$R_{kh} = \sum_{i=1}^{n} Z_{kih} R_{kih} \quad , \quad R_{kih} = y_{kih}/x_{kih} \qquad (2)$$

$$Z_{kih} = p_{kih} / \sum_{i=1}^{n} p_{kih} \quad , \quad \sum_{i=1}^{n} Z_{kih} = 1 \qquad (3)$$

$$Os_{kh}^{2} = n/q(n-1) \sum_{k=1}^{q} \sum_{i=1}^{n} Z_{kih}(R_{kih}-R_{kh})^{2} \qquad (4)$$

where k means animal, n=number of sections per animal, p=thickness of the stratum, Os_k^2 = variance between sections, q=number of animals. Otherwise, estimators were computed as usual.

Combination of the results from the different strata was carried out by using R_{ki} instead of R_{kih}:

$$R_{ki} = \sum_{h=1}^{3} W_{kih} R_{kih} \quad , \quad W_{kih} = p_{kih} / \sum_{h=1}^{3} p_{kih} \qquad (5)$$

When computing the numerical density of cells, the following estimator was used:

$$N_V = R_2/(D+t) \qquad (6)$$

where D=mean tangential diameter of nuclei, computed by volume equivalent sphere method using shape coefficients (Weibel, 1979); t=the section thickness.

RESULTS

The time budget was: (1) preparation of a condyle, about 40 min, (2) analysis of one section, about 15 min, and, (3) computing D from about 300 nuclear profiles per stratum, about 8 h.

Figs. 1 and 2 show the precision of estimators versus different number of blocks per animal. For example, six blocks per animal was enough to ensure the necessary precision of both animal and population estimators.

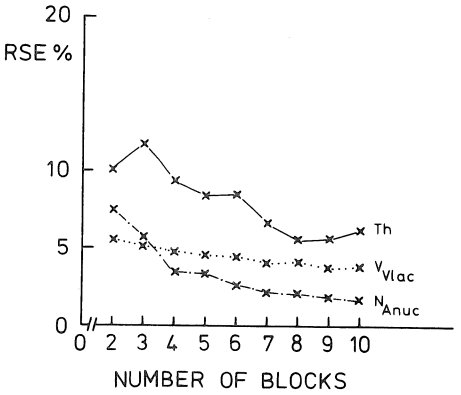

Fig. 1. Relative standard error of the mean (RSE_Q) of the population estimators versus different number of blocks per animal. Th=thickness, V_{Vlac}=volume density of lacunae, N_{Anuc}=numerical density of nuclei profiles.

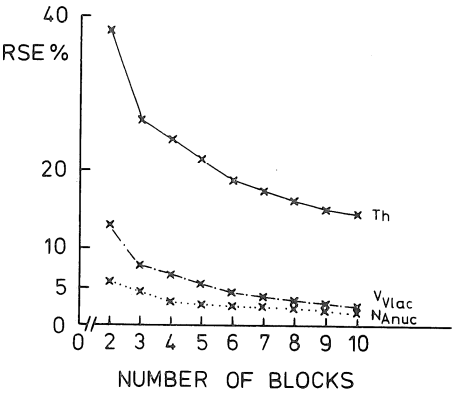

Fig. 2. Mean relative standard error of the mean (RSE_k) of the animal estimators plotted against different numbers of blocks per animal.

DISCUSSION

Stereology gives valuable tools for histomorphometric studies: mathematics for 3-d definition of structures, statistics for determination of the precision and the conditions for the validity of estimators.

In the stereology of articular cartilage, there are some practical difficulties, which confine the use of the methods based on the unbiased estimation theory (Miles and Davy, 1976). These limitations are due to the fact, that, when defining the structure of articular cartilage, also other morphometrical parameters than stereological ratio estimators, have to be estimated (e.g., cartilage thickness). In spite of the limitations, a uniform random sampling method, which allowed consideration of the precision and variation of the estimators also within one animal, could be designed. In addition, the estimators produced by the method could be assumed to be constant; they proved to be precise, too.

In our opinion, the efficiency of our sampling design became evident, when the precision of the estimators was compared to the rather small effort needed to gain a representative sample for reliable results.

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