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Evaluation of volume-weighted mean nuclear volume in endometrial lesions

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Summary. The individual nuclear size was studied in three groups of endometrial samples (simple hyperplasia -SH-, atypical hyperplasia -AH-, and well-differentiated adenocarcinoma -WDA-). The application of recent semiquantitative systems in combination with stereological methods permits a simple, quick and unbiased estimation of the volume-weighted mean nuclear volume $\overline{v}_V(nucl)$. We have found an increase in the $\overline{v}_V(nucl)$ from SH to AH to WDA. There are significant differences between the mean of the three groups ($p<0.01$). The variance associated with estimates of $\overline{v}_V(nucl)$ is mainly provided by differences among lesions; i.e. patients. This capacity for discriminating may be associated with gland and lumen endometrial quantification to improve the correct diagnosis of endometrial samples.

Key words: Endometrial lesions, Stereology, Point-sampled intercepts, Mean nuclear volume

Introduction

Diagnosis and gradation of pathological entities were usually performed by the subjective assessment of the pathologist. Lately, Hospital Pathology services have incorporated new methods (cytophotometry, immunohistochemistry, morphometry,...) to improve diagnostic reproducibility in several histopathological lesions. Specifically, endometrial samples for diagnosis have been widely studied (Ferenczy et al., 1983; Baak, 1984; Skaarland, 1985; Norris et al., 1989). Differentiation of both normal endometrial subphases and numerous pathological endometrial entities have been objectively classified using quantitative information (Kurman and Norris, 1982; Fu et al., 1988; Artacho-Pérula et al., 1992a,b). A high percentage of correct classification has been obtained using different quantitative parameters and choosing those which allow a better discrimination.

Quantitative analysis of the endometrium was mainly performed previously by determining the features of the glands, lumina and epithelium whereas few studies exist concerning nuclear size. Recent stereological methods have appeared which permit the easy, quick and unbiased determination of the nuclear volume (Gundersen and Jensen, 1985; Cruz-Orive, 1987; Jensen and Sorensen, 1991). Thus, the «point-sampled intercepts» method described by Gundersen and Jensen (1985) estimates the volume-weighted mean nuclear volume on individual histological sections. Its application to several histopathological entities has been sufficiently demonstrated in association with survival studies (Nielsen et al., 1986; Sorensen, 1989a).

The purpose of this study was to establish the mean nuclear volume in three groups of abnormal endometrium and to statistically evaluate the differences between these groups. The «point-sampled intercepts» method is also explained using a computer system developed by the University of Córdoba.

Materials and methods

Biological material

Thirty-six endometrial samples diagnosed as simple hyperplasia (SH; n=11), atypical hyperplasia (AH; n=9) and well-differentiated adenocarcinoma (WDA; n=16) were retrieved from the files of the Pathology Service of the Reina Sofía Hospital, Córdoba, Spain, between 1986 and 1987.

Histological sections (5 μm thick) were cut from each of the routinely processed, paraffin-embedded tissue blocks and stained with haematoxylin and eosin. Confirmation of the diagnosis was carried out by two pathologists following the criteria of Kurman et al. (1985) and described by us (Artacho-Pérula et al., 1992b).

Microscopy and computer systems

An Olympus BH-2 microscope modified by Bico

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A/S, Glostrup, Denmark, for stereological measurements was used. It was equipped with a 100 watt halogen light source to maximize its illuminating power, an electronic microcator for measuring vertical movements of the microscope stage, and a set of motors to systematically choose fields of vision through predeteminated «x» and «y» axes advances. An «object rotator» on the microscope stage permitted the rotation of the slide in the section plane. Attached to the microscope, a Hitachi colour camera (VK-C150ED) sent the microscopical image to a frame grabber board (DT2851) where it was captured and stored.

An IBM-compatible AT computer equipped with the frame grabber board and connected to two monitors was used. Imago software developed by the University of Córdoba was used for image processing. The microscopic image was displayed on a Multisync II colour monitor NEC while a monochrome monitor displayed the Imago programme for manipulation of the image. Following calibration of the system and improvement of image definition, different test systems previously acquired by the computer system for quantitative estimation of the nuclear volume were superposed.

Stereological analysis

A x40 objective lens was used for observation of the endometrial section and calibration of the system (using a millimetrical slicer). Once the endometrial slide was on the microscope stage, fields of vision were chosen systematically by using the motors incorporated into the microscope. Each chosen field was stored and displayed on the screen. Image definition was immediately improved. Thus, the user was prepared to measure the endometrial nuclei.

The stereological method for estimation of nuclear volume was described by Gundersen and Jensen (1985) and denominated the «point-sampled intercepts» method. The estimation of the volume-weighted mean nuclear volume $\bar{v}_v$*(nucl)* is easy, quick and simple. A test system characterized by a frame with points associated with lines which was previously stored on a hard disk catalogue was superposed on the microscopic image (Fig. 1). Each test point within the frame which hit a nucleus was used to determine the mean nuclear volume $\bar{v}_v$*(nucl)*: the linear intercept through the nuclei hit by test-points was measured in the direction of the associated test lines. This value was obtained automatically by consecutively placing the cursor on the two limits of each linear intercept. The averaged, cubed mean intercept length, $\bar{l}_0^3$. multiplied by $\pi/3$ is an unbiased estimate of $\bar{v}_v$*(nucl)* of the nuclei sampled with a chance proportional to their height.

Estimates of $\bar{v}_v$*(nucl)* are independent of nuclear shape. It is assumed that no preferential nuclear orientation was present in endometrial sections following approximate random orientation of tissue chips during the embedding procedure; thus indicating that routine sections for all intents and purposes are isotropic uniform random sections. This means that a fixed direction of the test lines may be used; i.e., it is not necessary to change the direction of the test lines for each field of vision and, consequently, we could use the

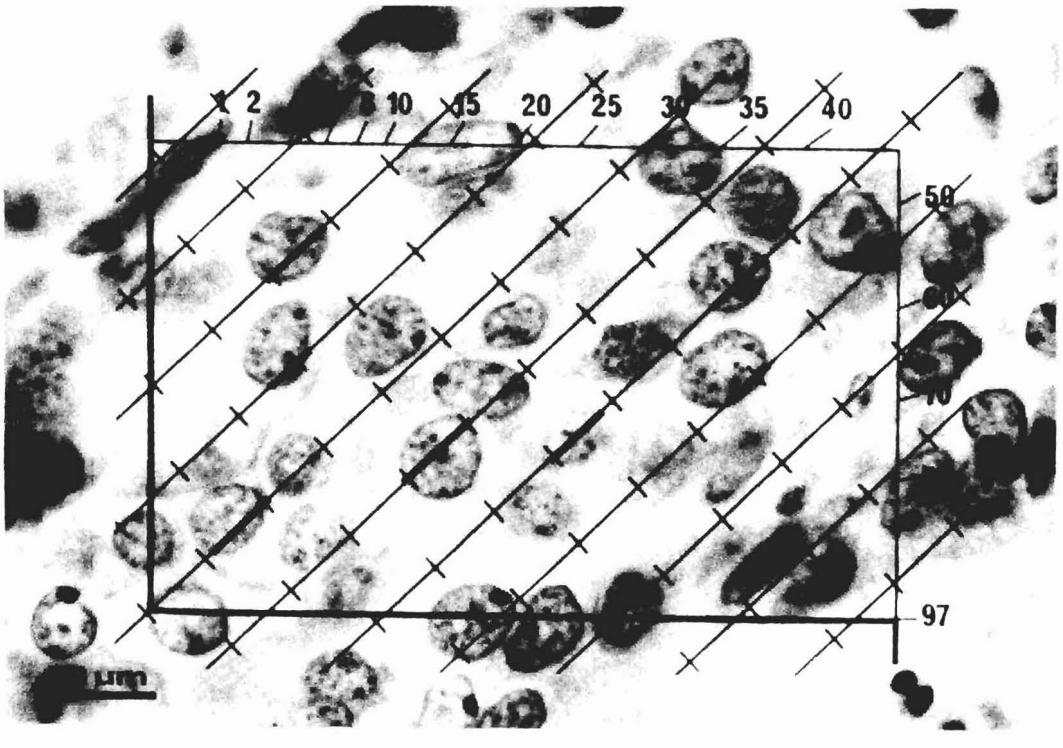

Fig. 1. Histological section from a well-differentiated adenocarcinoma. The «point-sampled intercepts» method is illustrated using a test system composed for an unbiased counting frame in conjunction with lines associated with test points. The test system was superimposed on the microscopic image to estimate volume-weighted mean nuclear volume. The thick segment represents nuclear intercept length measurement. H&E stain.

same test system for the entire study. The only requisite for obtaining unbiased stereological estimates of $\bar{v}_v(nucl)$ was the unambiguous identification of the particles - nuclei- and the user had to know if two or more profile transects belonged to the same particle or not.

The mean number of point-sampled nuclear intercepts was 95 per SH (range 75 to 158), 133 per AH (range 79 to 306), and 104 per WDA (range 77 to 185). On average 8, 8 and 6 fields of vision were studied for SH, AH and WDA respectively. A mean time consumption of 15 minutes was required per biopsy.

Statistical analysis

Analysis of variance was performed for comparing the mean values of $\bar{v}_v(nucl)$ from SH, AH and WDA. Homogeneity of variances was previously tested by the Cochrans C test (maximal variance/sum of variances). Multiple range test by the Scheffe procedure was used to detect the existence of group differences at levels of significance of $p<0.05$ and $p<0.01$.

The contribution to the total observed absolute variance of $\bar{v}_v(nucl)$ from the different levels of sampling (measurement of nuclear intercepts, different fields of vision and individual biopsies) was investigated by the method of nested analysis of variance (Gundersen and Osterby, 1981). Thus,

$$Os_b^2 = s_b^2 + (1/n_f) \cdot s_f^2 + (1/(n_f \cdot n_i)) \cdot (s_i^2 + s_m^2),$$

where s_b^2, s_f^2, s_i^2 and s_m^2 are the true variance at level of biopsies, fields of vision, intercepts and the variance added to the true variation between intercepts, respectively. n_f and n_i are the number of fields and intercepts per level just above them; and Os_b^2 is the observed variance at the highest level; i.e. biopsies.

All statistical analyses were performed on an IBM-PC compatible using an SPSS statistical package with means and oneway procedures.

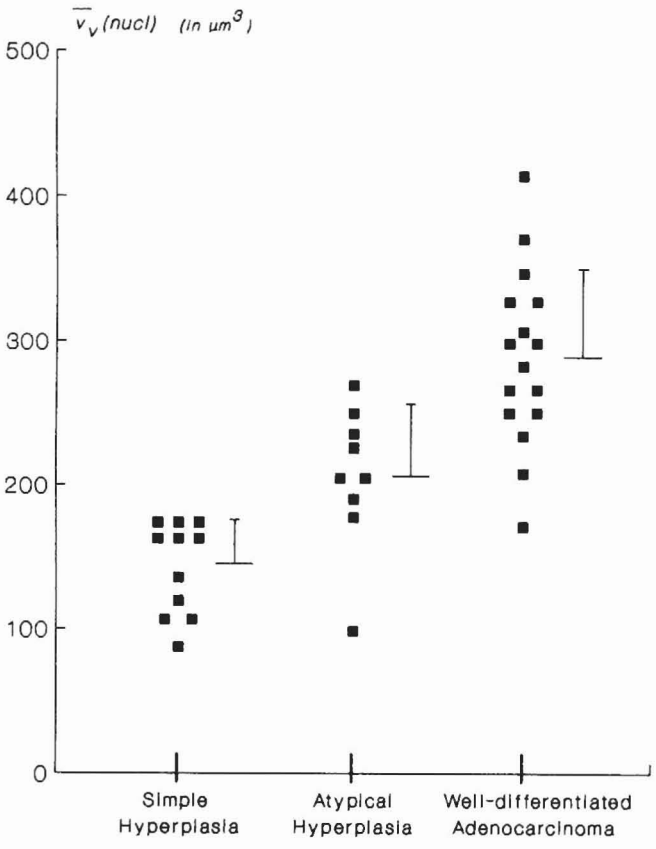

Fig. 2. Plot of $\bar{v}_v(nucl)$ for simple hyperplasia, atypical hyperplasia and well-differentiated adenocarcinoma. Horizontal bars indicate mean values; vertical bars indicate standard deviations.

Results

The quantitative estimates for $\bar{v}_v(nucl)$ of SH, AH and WDA are shown in figure 2 for each individual patient. A progressive increase existed from SH (group mean 142.7±33.3 μm^3) to AH (group mean 205.9 ± 50.1 μm^3) to WDA (group mean 288.3 ± 61.7 μm^3). Thus, values of $\bar{v}_v(nucl)$ greater than 270 μm^3 corresponded to WDA. All SH, two AH and only one WDA had $\bar{v}_v(nucl)$ less than 180 μm^3. The coefficients of error of the averaged $\bar{v}_v(nucl)$ were 7.0%, 8.1% and 5.4% for SH, AH and WDA respectively.

Statistical differences were found between the means of the three groups ($F=26.5$, $p<0.01$). Multiple range test denoted differences at a level of $p<0.05$ between WDA and AH, whereas statistical differences between SH and the other two groups were less than 0.01.

Figure 3 shows the distribution of $\bar{v}_v(nucl)$ for the three groups. The higher proportion of very small values of $\bar{v}_v nucl)$ corresponded to SH, whereas the higher porportion of very large values were present in WDA.

The contribution to the total variance from the variation in the measurements of individual intercepts, the variation from field to field and variation due to different true values of $\bar{v}_v(nucl)$ from patient to patient is illustrated in figure 4. The highest contribution to the overall variance was provided by differences between individual patients; i.e. biological variation (67.9%, 83.9% and 76.8% for SH, AH and WDA respectively). Both variation due to fields and intercepts were inferior to 18%.

Discussion

Previous studies (Baak et al., 1981, 1988; Artacho-Pérula et al., 1992b) have demonstrated the applicability of architectural measurements in discriminating endometrial pathology. Norris et al. (1989), using cytophotometric and morphometric estimators of the nuclear size and shape in combination with discriminat analysis, obtained a high relative discrimination between different entities. These authors found that the only parameter related to tissue architecture, the average epithelial cellularity, was the most powerful in discriminating endometrial entities. Norris et al. (1989) indicated that additional quantitative criteria on gland

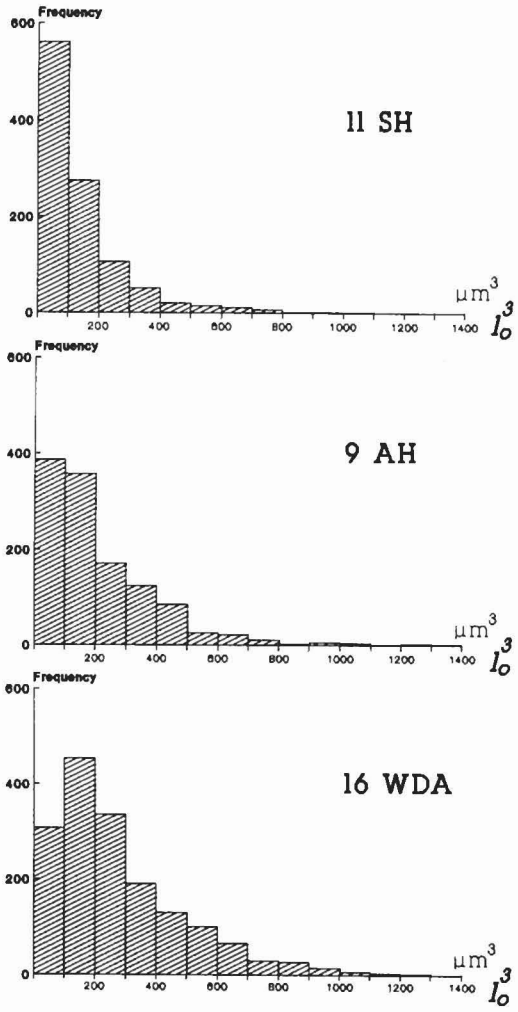

Fig. 3. Distribution of intercept length l_0^3 for three groups of endometrial samples.

architecture would be useful in improving the quantitative concordance in the classification of endometrial cases with respect to histopathological diagnosis.

The volume-weighted mean nuclear volume, a recently available parameter, achieves a good differentiation between SH, AH and WDA. This estimator provides three-dimensional information of the three-dimensional nuclei; and furthermore, this feature is clearly different for other morphometrical parameters. It is also different from conventional stereological methods which often give information in terms of ratios. In addition, estimates of $\overline{v}_V(nucl)$ provide special quantitative information of nuclear size in malignant disorders, since larger nuclei rather than smaller ones are sampled. Thus, estimates of $\overline{v}_V(nucl)$ are a powerful tool in the diagnostic and grading of different pathologies (Nielsen et al., 1986; Brüngger and Cruz-Orive, 1987; Howard et al., 1987; Sorensen, 1989a,b; Sorensen et al., 1989, 1991; Artacho-Pérula et al., 1992c) showing a high reproducibility (Nielsen et al., 1989).

The precision of the estimation of $\overline{v}_V(nucl)$ is acceptable in order to evaluate the nuclear size. Our study demonstrates a high contribution to the total variance associated with biological variation, i.e. patients, whereas the contribution to variance from different fields of vision and measurements of nuclear intercepts is smaller. This feature has been analyzed by numerous authors (Nielsen et al., 1986; Sorensen, 1989b; Artacho-Pérula et al., 1992c). Sorensen (1989b) observed no systematic enlargement of nuclei in the areas of skin lesions with the most pronounced cellular dedifferentiation in relation to all fields. The efficiency of the estimation of $\overline{v}_V(nucl)$, considering efficiency= 1/(time consumption x variance), has also been studied in endometrial samples showing a significant increase with respect to mean nuclear area quantification (Vaamonde-Lemos et al., 1992).

Different methods for measuring intercept lengths are

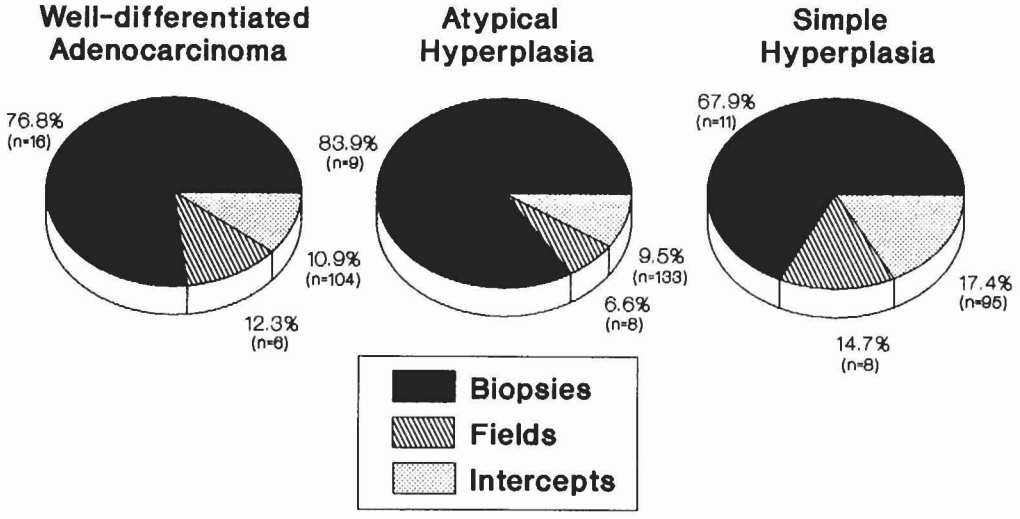

Fig. 4. Illustration of the relative contribution to the total observed variance from different parts of the method for estimation of $\overline{v}_V(nucl)$ in each endometrial group. Percentage of contribution is shown. Number of biopsies, average number of fields per biopsy and average number of intercepts measurements per biopsy are also registered.

at the disposition of researchers. It is interesting to know that for several years various types of rulers have been applied (Nielsen, 1991). Lately, the logarithmic l_o^3-ruler was used for classifying the intercepts in different classes (Braendgaard and Gundersen, 1986; Gundersen et al., 1988a,b). The use of software for calculation of intercept lengths permit an unbiased estimation of $\bar{v}_V(nucl)$. Two advantages are present when we use semi-automatic analyser in comparison with rulers: i) The suitable ruler length for measuring the major intercept need not be known; ii) The measurement is more rapid.

In conclusion, estimation of $\bar{v}_V(nucl)$ is a useful stereological tool for distinguishing between endometrial lesions. In combination with other quantitative parameters (two- and three-dimensional) referring to gland, lumen and epithelium it may improve the correct diagnosis and classification of different entities. This is an easy, simple and quick method for the unbiased estimation of nuclear size which only requires routine tissue processing, adequate calibration of magnification and systematic sampling of the biopsy.

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