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THE NUMBER OF METAPHASIC GRANULOSA CELLS IN ISOLATED OVARIAN FOLLICLES ESTIMATED USING THE NUCLEATOR PRINCIPLE

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ABSTRACT

Nine murine ovarian follicles embedded in plastic were orientated isotropically using the Orientator by Mattfeldt, cut into 20 μm serial sections and stained with hematoxylin. The number of granulosa cells in metaphase in each follicle was estimated using the Nucleator principle in the section which contained the nucleolus of the oocyte. The true number of granulosa cells in metaphase was obtained from the complete set of follicle sections using the 3-D counting rule. By knowing the truth, the precision (CE) of the Nucleator estimates could be calculated. The Nucleator provides sufficiently precise estimates of the small fraction of metaphasic granulosa cells. The estimates are unbiased and expressed in 3-D terms, thus making them suitable for statistical analysis and straight-forward biological interpretation.

Key words: Disector, follicle, granulosa cells, Nucleator, stereology.

INTRODUCTION

An ovarian follicle is histologically classified according to the appearance of its contents, i.e. oocyte, granulosa cells and follicular fluid forming the antrum, which changes as the follicle grows, matures or degenerates (Peters & McNatty, 1980). As the follicle possesses one easily recognizable and unique point-like unit, the nucleolus of the oocyte, it is ideal for unbiased stereological analysis using the Nucleator (Gundersen 1988, Gundersen et al., 1988b). We have recently shown, that the general Nucleator principle provides very precise estimates of the total three-dimensional

number of granulosa cells per follicle (Bagger et al., 1993) and of the volumes of follicle, oocyte and antrum (Bagger, 1993; Bagger et al., 1989). The aim of the present study was to evaluate the precision of the Nucleator with regard to estimation of a very small fraction of the granulosa cells, i.e. the number of granulosa cells with metaphasic nuclei (N_{Nu}metaphase).

MATERIAL AND HISTOLOGICAL PROCEDURE

Nine murine follicles (mean diameter = 154 μm, range 115 to 210 μm) fixed in 4% formalin were embedded separately in a drop of Agar, orientated isotropically using the Orientator principle (Mattfeldt, 1990; Bagger et al., 1993), embedded in plastic (LKB-glycolmethacrylate, Historesin^{R}), cut exhaustively into 20 μm thick (t = 20 μm), serial sections parallel to the cutting surface just established with the Orientator, and stained with hematoxylin. The sections were observed using a 100X (N.A. = 1.40) oil objective in a modified Olympus BH-2 microscope equipped with an electronic microcator (Haiderhain^{R}, VRZ 401) and a projection arm. For further details see Bagger (1993) and Bagger et al. (1993).

NUCLEATOR ESTIMATE

The section which contained the unique sampling point, the nucleolus of the oocyte, was projected onto the table. Metaphasic granulosa cell nuclei were sampled, when they came into focus, as the focal level was brought from 5 μm to 15 μm below the surface of the section (h = 10 μm); metaphases that were already in focus in the initial plane 5 μm below the surface of the section were not sampled. Finally, the distance from the nucleolus of the oocyte to each sampled metaphase (d) was measured, and

$$N_{Nu}\text{metaphase} = 2 \times \Sigma d / h \qquad (1)$$

provides an unbiased estimate of the number of metaphasic granulosa cells in the follicle; the constant 2 is specific for isotropically orientated sections (Gundersen et al., 1988b). The average number of metaphases sampled in this way constituted 1.0 metaphase per follicle (range 0 to 6).

TRUE NUMBER OF METAPHASES PER FOLLICLE

All sections through the follicles were inspected for metaphases, the number of which (N_{True}) were counted using the 3-D counting rule (Gundersen et al, 1988a).

STATISTICAL ANALYSIS

The analytical variation of the Nucleator principle, i.e the variability of the Nucleator estimates, was expressed as the (dimensionless) coefficient of error

$$CE(N_{Nu}) = (1/N_{True}) \times (\Sigma(N_{True} - N_{Nu})^2/n)^{1/2} \quad (2)$$

The above CE was compared to the expected CE based on the actual average count of cells, ΣQ^-:

$$E\{CE(N_{Nu})\} = 1 / (\Sigma Q^-)^{1/2} \quad (3)$$
$$E\{CE(N_{Nu}\text{metaphase})\} = 1 / 1^{1/2} = 1.0$$

which in this case assumes that ΣQ^- has a Poisson distribution. E{CE} is thus likely a lower limit for the real CE.

The biological variation, i.e. the variation between follicles, was calculated as

$$CV_{biol} = CV(N_{True}) = SD / N_{True} \quad (4)$$

where SD is between the nine N_{True}'s.

Other samples of nine follicles most probably have N_{Nu}'s different from one another and different from the N_{Nu} obtained in the present study. The statistical variation around the true group mean of the estimated sample means thus established is the sum of the variance within follicles (the analytical estimation variation) and the variance between follicles (the biological variation) (Fig. 1):

$$E\{CE_n(N)\} = ((CE^2(N_{Nu}) + CV^2(N_{True})) / n)^{1/2} \quad (5)$$

where n is the number of sampled follicles used for establishing the group mean (nine in the present example).

RESULTS

The number of metaphases estimated using the Nucleator was of the same order of magnitude as the true number (Tbl. 1). N_{True} and N_{Nu} correlated linearly to each other ($r=0.79$, $2P<0.02$). The analytical variation $CE(N_{Nu}\text{metaphase})$ of 0.85 was of the same order of magnitude as the expected $E\{CE(N_{Nu}\text{metaphase})\}$ of 1.0, which was equal to the CV_{biol}metaphase of 1.0.

Table 1. Number of metaphasic granulosa cells per follicle in nine murine ovarian follicles estimated using the Nucleator (N_{Nu}metaphase) compared to the true number of metaphasic granulosa cells (N_{True}metaphase). The average number of metaphases sampled for the Nucleator estimates constituted 1.0 metaphase per follicle, range 0 to 6.

	N_{True}metaphase	N_{Nu}metaphase
	30	21
	16	0
	10	0
	7	0
	6	0
	5	4
	3	4
	3	2
	1	0
Mean	9.0	3.4
1 SD	9.1	6.8
Mean difference		5.6
1 SEM		1.9
$CV(N_{True}) = CV_{biol}$	1.0	
$CE(N_{Nu})$		0.85
$E\{CE(N_{Nu})\}$		1.0
$E\{CE_g(N)\} =$		0.44
$CV(N_{Nu})$ = observed $CE_g(N)$		0.66

DISCUSSION

In the current study we have determined the precision of the Nucleator with regard to estimation of a very small number of cells within the ovarian follicle, the number of metaphasic granulosa cells per follicle, which in these nine follicles constituted 2 ‰ of the granulosa cells (the average number of granulosa cells per follicle in these nine follicles was ca. 4,700, see Bagger et al., 1993). We find that even though the estimate is statistically less precise ($CE(N_{Nu}) = 0.85$), it is of the same order of magnitude as the truth (N_{Nu}metaphase = 3.4 ± 6.8; N_{True}metaphase = 9.0 ± 9.1).

Thus, for practical purposes the Nucleator may provide acceptably precise, unbiased estimates of very small fractions of granulosa cells, e.g. granulosa cells in metaphase and granulosa cells with pyknotic nuclei. The estimates are unbiased, because embedding and cutting are performed in random positions and orientation, for

example, using the Orientator (Mattfeldt et al., 1990) or the Isector (Nyengaard & Gundersen, 1992), and because of uniform, systematic random sampling. They are unbiased, irrespective of the shape of the follicle, the size and shape of the granulosa cells, and the position of the oocyte nucleolus. The estimates are obtained with a minimal amount of effort, since sampling is performed in only one section through the follicle, the section that contains the unique sampling point, the nucleolus of the oocyte. Moreover, it is not necessary to know the thickness of the section, and one follicle can be measured in two minutes, obviously almost ideal conditions for most studies.

The analytical variation depends on the position of the oocyte nucleolus, the shape of the follicle, and on the number of metaphasic granulosa cell nuclei sampled (ΣQ^-, eq.s 2 and 3), i.e. $E\{CE(N_{Nu})\}$ in eq. 3 is inversely correlated to ΣQ^-. In the present study the real CE of 0.85 is of the same order of magnitude as the expected one of 1.0. This indicates that ΣQ^- is the most important source of variation. It is also the only source of variation that ordinarily can be influenced by the investigator. Decreasing analytical variation by sampling more cells demands a sufficient quantity of cells. The possibility of decreasing analytical variation by sampling more cells depends on the total number of cells with a chance of being sampled, i.e. estimating small quantities of cells reduces this possibility proportionally.

Analytical ($CE(N_{Nu}) = 0.85$) and biological variation ($CV_{biol} = 1.0$) are of equal importance in determining the variation of the average number of metaphasic granulosa cells per follicle in a group of follicles ($E\{CE_n(N)\}$, eq. 5); it is assumed that the conditions for using the Nucleator are fulfilled, and that the follicles in this context are sampled in a systematic uniform way (Bagger et al., 1993). Neither $CE(N_{Nu})$ nor CV_{biol} can be influenced to any greater extent during sampling. Increasing fourfold the number of follicles investigated to 36 decreases $E\{CE_n(N)\}$ to half of 0.44, i.e. a negligible improvement considering that the total number of metaphases per follicle is 9.

In conclusion, the Nucleator provides sufficiently precise estimates of tiny fractions of granulosa cells within the ovarian follicle. The estimates are unbiased and expressed in 3–D terms, thus making them suitable for statistical analysis and a sufficient basis for drawing biological conclusions.

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