Single-section counting error when distinguishing between primordial and early primary follicles in sections of rat ovary of different thickness

S. Meredith*, G. Dudenhoeffer and K. Jackson

Cooperative Research, Lincoln University, Jefferson City, MO 65102-0029, USA

The number of primordial follicles within an ovary is frequently determined by counting 5, 7 or 10 μm thick sections and multiplying by the fraction of sections counted and a correction factor to adjust for duplicate counts. The objectives of the present study were: (i) to evaluate the accuracy of the correction factor developed by Abercrombie (1946); (ii) to evaluate the accuracy of the classification of primordial follicles from single tissue sections; and (iii) to determine the incorporation rate of 5-bromo-2-deoxyuridine into primordial follicles. In Expt 1, rat ovaries were sectioned at a thickness of 5, 7 or 10 μm . Primordial follicles were counted and classified across ten adjacent ovarian sections. The percentage of primordial follicles from single sections that were counted twice was 10, 9 and 2% in 5, 7 and 10 μm sections, respectively. This was lower than predicted by Abercrombie's method. The major error in counting from single sections was classification of early primary follicles as primordial follicles (55, 33 and 3% in 5, 7 and 10 μm sections, respectively). In Expt 2, a mean of $12 \pm 7\%$ of primordial follicles incorporated 5-bromo-2-deoxyuridine after infusion for 7 days (four of seven rats had no labelled primordial follicles). In conclusion: (i) Abercrombie's correction factor should not be used for adjusting counts of follicles; (ii) evaluation of primordial follicles from single sections gives inaccurate counts and incorrect classification is of greater importance than duplicate counting, particularly in thinner sections; (iii) for evaluation of the number of follicles, 10 μm is the optimal thickness; and (iv) primordial follicles incorporated 5-bromo-2-deoxyuridine infrequently.

Introduction

Primordial follicles are formed early in life, and after this period *de novo* formation does not occur. Therefore, the pool of primordial follicles forms the lifetime supply of oocytes and, once depleted, reproduction ceases. In humans, there is a slow and steady reduction in the number of primordial follicles until a mean age of 37.5 years (Faddy *et al.*, 1992). After this age, the rate of loss of primordial follicles increases, causing rapid depletion which culminates in the menopause (Faddy *et al.*, 1992; Faddy and Gosden, 1995). After menopause, many deleterious processes occur such as memory loss (Kampen and Sherwin, 1994), increased risk of cardiovascular disease (Mijatovic *et al.*, 1997; Nasr and Breck Woldt, 1998), increased risk of osteoporosis (Riggs *et al.*, 1998) and decrease in antioxidant activity (Green *et al.*, 1998). All of the processes can be attributed, at least in part, to a reduction in oestrogen activity. Understanding the mechanisms involved in activation and depletion of primordial follicles might enable the number of primordial follicles to be manipulated, causing

a delay in the onset of the menopause, and therefore a delay in the decrease of endogenous oestrogen.

Before the mechanisms involved in the activation of primordial follicles can be understood, it is necessary to determine the accuracy of methods that are used frequently for evaluation. Normally, in rats, the intact ovary is sectioned and a proportion (for example, 1:10) of the sections are evaluated. The thickness of the sections is typically in the range 2–10 μm (Abercrombie, 1946; Meredith and Butcher, 1985; Hirshfield, 1989; Meredith and Doolin, 1997). When ovaries are sectioned in this manner and the nucleolus is used as the unique identifier, the likelihood of duplicate counting and thus overestimation of the number of follicles is low. This is because the nucleolus has a small diameter relative to the thickness of the sections.

The nucleus is sometimes used as the unique identifier rather than the nucleolus (Abercrombie, 1946; Meredith and Doolin, 1997). The likelihood of duplicate counting is greater when the nucleus is used because of its larger size. Abercrombie (1946) developed a mathematical adjustment for counts, which can be summarized as: section thickness / (section thickness + diameter of the nucleus). The basis of this formula is that the object is spherical and is visible in all

*Correspondence address: 306A Foster Hall, Lincoln University, Jefferson City, MO, 65102-0029, USA.

Revised manuscript received 26 April 1999.

sections containing at least a portion of the object. It is possible that neither of these assumptions is correct, and one purpose of this study was to use the nucleus as the unique identifier to determine the frequency at which each follicle would be counted on adjacent tissue sections.

Primordial and early primary follicles should be distinguished when studying follicular activation since they appear to be physiologically distinct groups (Oktay *et al.*, 1995; Wandji *et al.*, 1995). Distinguishing primordial and early primary follicles on a single section results in a second source of error because follicles classified as primordial in one section may be observed in adjacent sections to contain cuboidal granulosa cells. The presence of only a single cuboidal cell in an adjacent section would result in incorrect classification of an early primary follicle as a primordial follicle. The frequency of incorrect classification is likely to be higher in thin sections because a smaller percentage of granulosa cells are visible compared with thick sections.

Immunohistochemistry is a common method for determining the proportion of follicles with granulosa cells undergoing DNA synthesis. It is likely that previous work showing the percentage of small follicles labelled (37%) with [³H]thymidine after infusion for 7 days (Hirshfield, 1989) represents a combination of primordial and early primary follicles. It is possible that only a small proportion of the 37% are actually primordial follicles.

These potential counting and classification problems can be determined experimentally by evaluating primordial follicles on the section of interest and examining adjacent sections for the same follicle and its associated granulosa cells. This allows the frequency at which early primary follicles are classified as primordial follicles in the initial section to be determined. The objectives of the present study were to determine: (i) the validity of Abercrombie's correction factor; (ii) the frequency at which incorrect classification of primordial follicles occurs in single sections of different thickness; and (iii) the frequency of uptake of 5-bromo-2-deoxyuridine (BrdU) in primordial and early primary follicles.

Materials and Methods

General

Holtzman rats were obtained from our colony at weaning (rats were originally purchased from Harlan Sprague-Dawley, Inc., Indianapolis, IN) and maintained under controlled conditions of temperature (22°C), humidity and lighting (lights on at 06:00 h and lights off at 18:00 h). Ovaries were removed and placed immediately in Carnoy's fixative for 2 h, after which they were transferred to 70% alcohol until processed for histological evaluation.

Throughout this study, primordial follicles were defined as an oocyte surrounded only by flattened granulosa cells; early primary follicles were defined as oocytes surrounded by a mixture of flattened and cuboidal granulosa cells; and primary follicles were defined as oocytes surrounded by a single complete layer of cuboidal granulosa cells (Oktay *et al.*, 1995).

Experiment 1

Females were killed by CO₂ inhalation at 27 days of age (day 0 = day of pupping) and the ovaries were removed. A total of 18 ovaries were embedded in paraffin wax after fixation and divided equally into three groups. Groups 1, 2 and 3 represent serial sections of ovaries made at a thickness of 5, 7 and 10 µm, respectively. The ovaries were sectioned stained with haematoxylin and eosin, and the sections were examined through a microscope to locate a relatively dense region of small follicles. Once this was located, photomicrographs were taken of ten consecutive sections (see Fig. 1a for a side view of the counting area and Fig. 1b for a top view of the sections). Colour prints were developed and photocopied to transparencies so each follicle could be evaluated on adjacent sections. Beginning with section 2, all follicles that matched the classification of primordial follicles were counted and each adjacent section was evaluated (that is, transparencies for section 1 and section 3). If the follicle from section 2 was found on either of the adjacent sections, the granulosa cells were evaluated to determine whether any were cuboidal. If they were, the follicle was considered an early primary follicle that was initially incorrectly classified as a primordial follicle. Classifications continued in this manner up to section 9. New follicles in section 10 were not evaluated.

Experiment 2

The objective of this experiment was to determine the percentage of primordial and early primary follicles containing granulosa cells that were undergoing DNA synthesis during infusion of BrdU for 7 days (Aldrich Chemical Company, Milwaukee, WI). Holtzman rats ($n = 7$; 48 days of age) were anaesthetized with ether and 7 day Alzet osmotic minipumps were implanted s.c. between the shoulder blades. Before insertion, pumps were filled with a 20 mg ml⁻¹ solution of BrdU dissolved in 0.9% sterile saline and incubated for 2 h in a container of 0.9% sterile saline. Rats were killed by CO₂ inhalation 7 days after insertion of the minipumps; the uterus was examined for ballooning; and the ovaries were removed. Ovaries were fixed as described for Expt 1, and sectioned serially at 7 µm. The procedure for immunohistochemistry was as described by Meredith and Doolin (1997).

The number of follicles was determined in sections (range 3–10 serial sections) as described in Expt 1, except that follicles surrounded by granulosa cells incorporating BrdU (labelled follicles) were distinguished from unlabelled follicles.

Statistical analysis

Data were analysed by one-way ANOVA. Significant differences among means were determined using least square means procedure when appropriate (Wilkinson, 1990). Differences were considered significant when $P < 0.05$. All data are expressed as mean $\pm$ SEM.

Fig. 1. (a) Schematic representation of a side view of the ten serial sections (stacked sections) of rat ovary. The number and classification of follicles were determined on each individual section as would normally be the case when evaluating only one of every ten sections. Each section was evaluated with adjacent sections to obtain counts of follicles and to determine the error in initial classification of early primary follicles as primordial follicles. For example, section 2 was evaluated and the number of primordial follicles in the section was determined. Sections 1 and 3 were subsequently evaluated for two purposes: (i) to determine whether the same follicles were visible in either section 1 or 3; and (ii) if the follicle was visible, to determine whether the surrounding granulosa cells were cuboidal. This procedure was repeated for the other sections up to section 9, which was the last section evaluated. Incorrect classification would occur if follicle A was only evaluated in section 2, follicles B and C only in section 6, or follicle D only in section 9. (b) A top view of the rat ovarian section when comparing follicles between two transparency films after making colour copies from photographs. The solid circles represent follicles observed on the top transparency film and the circles of dashed lines represent follicles observed on the middle transparency film. For purposes of clarity, the third transparency film in this series is not shown. The part labelled A represents a landmark on the ovary that was used to align the small follicles. The large square represents the area included in the photographs of the ovary. The follicles marked b were seen on both sections and would be considered follicles that were counted twice. The follicles marked c were not found in the top transparency film and were not considered to be counted twice. Follicles found only on the top section (d) were not counted.

Results

Only 10% of primordial follicles were counted twice in 5 μ m sections, when the nucleus was used as the unique identifier for the follicle (Table 1). This was not different ($P > 0.05$) from the extent of duplicate counting in sections of 7 and 10 μ m. When all counted follicles (including those that were classified as primordial in the initial section but were determined to be early primary follicles from the adjacent sections) were evaluated, duplicate counting was high and was affected significantly by the thickness of tissue sections (Table 1). This was not due to an increase in the diameter of the nucleus because there was no difference ($P > 0.05$) in the size of the nucleus between primordial ($9.2 \pm 0.22 \mu$ m) and early primary follicles ($9.5 \pm 0.22 \mu$ m).

Primordial follicles were classified correctly in individual 5 μ m sections less frequently ($P < 0.0001$) than in 7 or 10 μ m sections (Table 2). Applying Abercrombie's calculations to the data, subtracting the results from one and multiplying by 100 to convert to percentage values, resulted in predicted duplicate counting estimates of 65, 57 and 48% for 5, 7 and 10 μ m sections, respectively.

Experiment 2

The use of transparencies to identify the same follicles in adjacent sections (as described for Expt 1) resulted in $12 \pm 7\%$

(range 0–37; four of seven animals had no labelled primordial follicles) and $24 \pm 8\%$ (range 0–68; one of seven rats had no labelled early primary follicles) of primordial and early primary follicles, respectively, that were labelled with BrdU during infusion for 7 days.

Discussion

It was expected that counting all follicles when the nucleus was present would result in correction factors of 35, 43 and 52% for 5, 7 and 10 μ m sections, respectively. These figures were derived from the formula published by Abercrombie (1946).

Abercrombie's method is assumed to account for the overestimation of the number of primordial follicles after counting on single ovarian sections. This is because it is impossible to section the ovary without fragmentation of some follicles. Fragmentation results in some follicles being counted as new follicles on adjacent sections. The proportion of follicles that are fragmented increases as the diameter of the follicle increases relative to the thickness of the section. Assuming a section thickness of 7 μ m, large follicles would be fragmented more frequently than small follicles and therefore these would be counted in more sections (that is, a volume-weighted probe). Abercrombie (1946) was aware of this problem and developed a mathematical correction to be

Table 1. Effect of thickness of rat ovarian sections on percentage of follicles initially classified as primordial that were counted twice

Thickness of section (μm)	n^a	Percentage of total number of follicles that were counted twice ^b	Percentage of true primordial follicles that were counted twice ^c
5	6	56 ± 5^d	10 ± 6^g
7	6	23 ± 5^e	9 ± 4^g
10	6	3 ± 2^f	2 ± 2^g

^aNumber of ovaries analysed.^bThis column includes follicles that were classified as primordial on the initial section, but were re-classified as early primary follicles because a cuboidal granulosa cell was observed on an adjacent section.^cThis column includes follicles that were classified as primordial on the initial section and retained the classification of primordial when examined on adjacent sections. This column therefore represents the frequency with which primordial follicles were counted twice.Values within columns with different superscripts are significantly different ($P < 0.0001$).**Table 2.** Effect of thickness of rat ovarian sections on percentage of follicles that were correctly classified as primordial from single sections

Thickness of section (μm)	n^a	Primordial follicles classified correctly (%)	Range per ovary (%)
5	6	45 ± 6^b	25–63
7	6	77 ± 6^c	60–100
10	6	97 ± 2^d	92–100

^aNumber of ovaries analysed.Values with different superscripts are significantly different ($P < 0.0001$).

applied to counts. This correction factor accounted for the size of follicles relative to the thickness of the section and was based upon the assumption that follicles could be identified and counted in every section in which they were present. However, it is unlikely that fragmented primordial follicles are always visible in more than one section. The inability to observe portions of the cell of interest is sometimes referred to as lost caps (Gundersen, 1986). By evaluating follicles on adjacent sections in the present experiment it was demonstrated that duplicate counts of primordial follicles occur much less frequently than would be expected on the basis of size alone, and shows that primordial follicles would be counted on more than one section infrequently.

The major error in counting is not duplicate counting but incorrect classification of early primary follicles as primordial follicles. Early primary follicles initially classified as primordial were counted twice at a higher frequency than primordial follicles, even though there was no significant difference in the diameter of the nucleus. This difference appears to be due to the technical difficulty of identifying follicles when the nuclei, but not the granulosa cells, are visible. Follicles were counted on adjacent sections only when they were clearly identifiable as follicles and the nucleus was present. Since the total diameter of early primary follicles is slightly greater than that of primordial follicles (Oktay *et al.*, 1995), granulosa cells were visible on more than one section more frequently in early primary compared with primordial follicles. This means that lost caps (the inability to locate the nucleus when it is actually present in the section) occurred at a greater frequency in primordial than early primary follicles.

The results of the present study cast doubt on other studies in which attempts have been made to establish the relative number of primordial follicles during the perimenopausal period (Richardson *et al.*, 1987), or to evaluate the percentage of primordial follicles that expresses proliferating cell nuclear antigen (PCNA) (Oktay *et al.*, 1995), or incorporates [³H]thymidine (Hirshfield, 1989) or BrdU (Meredith and Doolin, 1997). The results of the present study demonstrate that exhaustive sectioning of the ovary and evaluation of individual tissue sections when either the nucleus or the nucleolus is present results in inaccurate classification of follicles. This is because individual tissue sections, particularly thinner tissue sections, do not allow visualization of all granulosa cells surrounding an oocyte. Relatively accurate counts were obtained in the present study using 10 μm sections. A disadvantage of using 10 μm sections is the lack of clarity and the inability to use many of the immunohistochemical techniques available. It also is probable that evaluation of single 10 μm sections will not result in accurate determination of the relative numbers of primordial and early primary follicles in species in which these follicles have a greater diameter.

The classification errors reported in this paper could differ under other experimental conditions because of changes in the proportion of primordial and early primary follicles that are likely to occur with different treatments, ages or strains of rats. These results simply illustrate the source of major errors when evaluating primordial and early primary follicles and support the proposal that the total number of primordial and early primary follicles should be determined by other stereology methods such as the disector or

fractionator (Stereo, 1983; Gundersen, 1986; Miller *et al.*, 1997).

Hirshfield (1989) reported that 37% of follicles with four or fewer granulosa cells in the largest cross-section incorporated [³H]thymidine after infusion for 7 days. Although these were classified differently than in the present experiment, they would probably be a mixture of primordial and early primary follicles. In Expt 1 in the present study it was demonstrated that in evaluations of single sections, many early primary follicles were classified as primordial follicles.

The hypothesis for Expt 2 was that granulosa cells from early primary, but not primordial, follicles undergo DNA synthesis. BrdU should give results similar to [³H]thymidine (Qin and Willems, 1993). In Expt 2, the majority of rats had no primordial follicles with labelled granulosa cells. The few primordial follicles that contained squamous granulosa cells expressing BrdU may have been in the process of conversion to early primary follicles. Therefore, this technique still resulted in some labelled primordial follicles after one week of infusion with BrdU, but the percentage of labelled follicles was low. This demonstrates that granulosa cells of primordial follicles incorporate BrdU infrequently, even after prolonged periods of infusion. Therefore, these results are in partial agreement with the lack of PCNA expression in primordial follicles (Oktay *et al.*, 1995).

Twice as many early primary follicles as primordial follicles incorporated BrdU. This rate of labelling is not as great as reported by Hirshfield (1989) in small follicles, but shows that many of the small follicles evaluated by Hirshfield would probably have included a substantial number of early primary follicles. The rate of labelling for early primary follicles in the present study is similar to those identified at this age using only single tissue sections (S. Meredith, unpublished).

A remaining question is whether the conversion of primordial to early primary follicles is a marker for activation followed by immediate and continuous growth of the follicle. Appearance of PCNA (Oktay *et al.*, 1995; Wandji *et al.*, 1996), or uptake of [³H]thymidine (Hirshfield, 1989) or BrdU (present paper) appears to be correlated with the transformation of primordial follicles to early primary follicles. From studies in women (Gougeon and Chainy, 1987) and cattle (van Wezel and Rodgers, 1996), it is questionable that, once formed, early primary follicles develop immediately. This is primarily because these follicles constitute the largest compartment within the ovary (82.5% in cattle, van Wezel and Rodgers, 1996; 85.5% in rats, S. Meredith, unpublished), and the ovary would soon be depleted of follicles if these began to grow immediately. Therefore, it appears likely that both primordial and early primary follicles comprise the pool of primordial follicles. Whether it is necessary to distinguish between them will depend upon the experiment.

It is concluded that: (i) Abercrombie's correction factor should not be used because it does not result in properly corrected counts; (ii) sections adjacent to the follicle of interest must be evaluated in 5 and 7 µm sections to distinguish primordial from early primary follicles accurately; (iii) 10 µm sections result in more accurate counts

than 5 or 7 µm sections; and (iv) BrdU was not incorporated into primordial follicles after infusion for 7 days in most rats in the present study.

Paper no. S4-104-99 of the Lincoln University Cooperative Research Program and supported by NIH-MBRS grant S06-GM-08202-15, USDA MOX-OP97-417 and CSREES MOX-OP97-417.

References

- Abercrombie M (1946) Estimation of nuclear population from microtome sections *Anatomical Record* **94** 239–247
- Faddy MJ and Gosden RG (1995) A mathematical model of follicle dynamics in the human ovary *Human Reproduction* **10** 770–775
- Faddy MJ, Gosden RG, Gougeon A, Richardson SJ and Nelson JF (1992) Accelerated disappearance rate of ovarian follicles in mid-life: implications for forecasting menopause *Human Reproduction* **7** 1342–1346
- Gougeon A and Chainy GBN (1987) Morphometric studies of small follicles in ovaries of women at different ages *Journal of Reproduction and Fertility* **81** 433–442
- Green PS, Gridley KE and Simpkins JW (1998) Nuclear estrogen receptor-independent neuroprotection by estratrienes: a novel interaction with glutathione *Neuroscience* **84** 7–10
- Gundersen HJG (1986) Stereology of arbitrary particles. A review of unbiased number and size estimators and the presentation of some new ones, in memory of William R. Thompson *Journal of Microscopy* **143** 3–45
- Hirshfield AN (1989) Granulosa cell proliferation in very small follicles of cycling rats studied by long-term continuous tritiated thymidine infusion *Biology of Reproduction* **41** 309–316
- Kampen DL and Sherwin BB (1994) Estrogen use and verbal memory in healthy postmenopausal women *Obstetrics and Gynecology* **83** 979–983
- Meredith S and Butcher RL (1985) Role of decreased numbers of follicles on reproduction performance in young and aged rats *Biology of Reproduction* **32** 788–794
- Meredith S and Doolin D (1997) Timing of activation of primordial follicles in mature rats is only slightly affected by fetal stage at meiotic arrest *Biology of Reproduction* **57** 63–67
- Mijatovic V, Kenemans P, Netelenbos JC, Peters-Muller ER, van Kamp GJ, Voetberg GA, van de Weijer DH and van der Mooren MJ (1997) Oral 17-beta estradiol continuously combined with progesterone lowers serum lipoprotein(a) concentration in healthy postmenopausal women *Journal of Clinical Endocrinology and Metabolism* **82** 3543–3547
- Miller PB, Charleston JS, Battaglia DE, Klein NA and Soules MR (1997) An accurate, simple method for unbiased determination of primordial follicle number in the primate ovary *Biology of Reproduction* **56** 909–915
- Nasr A and Breck Woldt M (1998) Estrogen replacement therapy and cardiovascular protection: lipid mechanisms are the tip of an iceberg *Gynecology and Endocrinology* **12** 43–59
- Oktay K, Schenken RS and Nelson JF (1995) Proliferating cell nuclear antigen marks the initiation of onset of follicular growth in the rat *Biology of Reproduction* **53** 295–301
- Qin Y and Willems G (1993) Comparison of the classical autoradiographic and the immunohistochemical methods with BrdU for measuring proliferation parameters in colon cancer *Anticancer Research* **13** 731–735
- Richardson SJ, Senikas V and Nelson JF (1987) Follicular depletion during the menopausal transition: evidence for accelerated loss and ultimate exhaustion *Journal of Clinical Endocrinology and Metabolism* **65** 1231–1237
- Riggs BL, Khosla S and Melton LJ, III (1998) A unitary model for involutional osteoporosis: estrogen deficiency causes both type I and type II osteoporosis in postmenopausal women and contributes to bone loss in aging men *Journal of Bone and Mineral Research* **13** 763–773
- Stereo DC (1983) The unbiased estimation of number and sizes of arbitrary particles using the disector *Journal of Microscopy* **134** 127–136
- van Wezel IL and Rodgers RJ (1996) Morphological characterization of bovine primordial follicles and their environment *in vivo*. *Biology of Reproduction* **55** 1003–1011
- Wandji S-A, Srsen V, Voss AK, Eppig, JJ and Fortune JE (1996) Initiation *in vitro* of growth of bovine primordial follicles *Biology of Reproduction* **55** 942–948
- Wilkinson L (1990) SYSTAT: The System for Statistics Evanston, IL: SYSTAT, Inc.