

Debate

Ovarian follicle counts – not as simple as 1, 2, 3

Jonathan L Tilly*

Address: Vincent Center for Reproductive Biology, Department of Obstetrics and Gynecology, Massachusetts General Hospital/Harvard Medical School, Boston, Massachusetts 02114, USA

Email: Jonathan L Tilly* - jtilly@partners.org

* Corresponding author

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The functional life span of the ovaries is dictated in large part by the number of oocytes present, a number that is known to decline precipitously during both fetal development and postnatal life. Furthermore, studies from numerous laboratories have shown that exposure of the ovaries to a variety of pathological insults, such as anti-cancer therapies and environmental toxicants, accelerates oocyte and follicle depletion, consequently hastening the time at which ovarian failure is observed. Accordingly, over the past several years a tremendous amount of research effort has been expended to uncover the genetic and molecular mechanisms responsible for determining the size of the follicle reserve endowed at birth as well as the rate at which this stockpile of follicles is subsequently depleted [reviewed in [1,2]]. Since, however, there are no known serum markers that can be used to accurately estimate the number of follicles in the ovaries – in particular follicles at the earliest stages of development (primordial, primary, small preantral) – some type of histomorphometric evaluation of the ovary is generally employed.

Although conceptually this sounds like a rather straightforward, albeit tedious, procedure, the total number of follicles reportedly present in the ovaries of a particular species at any given time in life can vary by 10-fold or more, depending on the study. In fact, Pepling and Spradling [3] recently commented that "reports of germ cell number in fetal and neonatal mouse female gonads have varied from 3,500 to 30,000 but these experiments utilized different strains, different developmental times, and different methods". These authors went on to state that "further studies will be required to confirm the reality of such a large variation in the number of germ cells in dif-

ferent strains" [3]. Such studies, using one outbred and five inbred mouse strains, have recently been completed [4]. It was demonstrated that the total number of follicles present in neonatal life, as well as the size of the primordial follicle reserve, does vary depending on strain. In fact, up to 2-fold differences in follicle numbers between different strains was observed [4]. However, as striking as this finding is, it does not account for even one-quarter of the 10 (or more)-fold variation in absolute follicle numbers reported by different laboratories studying the mouse ovary.

Probably the most widely used approach for estimating follicle numbers is one based on a histological sampling of the total ovarian mass for the number of primordial, primary and preantral follicles, often followed by the application of a 'correction factor'. This approach essentially entails that an ovary is fixed, paraffin-embedded and serially sectioned, usually at 6–8 μm widths. The serial sections are placed in order on glass microscope slides and stained with a vital dye. Depending on the study, every fifth to every tenth section is then analyzed by light microscopy for the presence of primordial, primary and preantral follicles. The starting section is usually selected randomly (for example, if one is counting follicles in every fifth section, any one of the first five sections could be used to start the process), and only those follicles in which the nucleus of the oocyte is clearly visible are scored. Assuming a random distribution of follicles at the various stages of development throughout the ovary, a close estimate of total follicle numbers per ovary can theoretically be obtained if the ovarian sections not included in the analysis are accounted for (see below). How reliable is this technique? A paper by Hirshfield and Midgley [5]

published 25 years ago directly tested the accuracy of estimating the number of follicles of various size categories in the adult rat ovary by sampling every fifth section versus the actual number derived from counting follicles in every section. For those values obtained by evaluating every fifth section, the cumulative follicle counts were multiplied by a factor of 5 to account for the fact that four-fifths of the ovary was not analyzed. After studying the results, the authors concluded, "the discrepancy between the two estimates was so great that this method [sampling every fifth section] was discarded because it was so inaccurate" [5].

This conclusion is somewhat puzzling, however, based on the fact that the estimated number of total follicles, derived from sampling every fifth section, varied from the actual number of total follicles by only 3–11% [5]. Furthermore, subsequent studies published by Hirshfield and colleagues, which describe results from the assessment of follicle numbers in mice, utilized the procedure of sampling every fifth [6] or every tenth [7] section. Accordingly, the approach of sampling a fraction of the ovary appears sound and accepted, a conclusion reinforced by a series of experiments published in 1997 from a collaborative effort between the National Center for Toxicological Research and the National Institute of Environmental Health Sciences [8,9]. Assuming, then, that the sampling procedure provides a reliable estimate of the number of follicles per ovary, there remains an issue that is probably the principal cause of the large discrepancy in follicle numbers reported by various laboratories. This issue concerns the correction factor that is used to account for that proportion of the ovary not included in the sampling analysis.

For example, in 1978 Hirshfield and Midgley used a correction factor of 5 in their studies that examined every fifth section of the ovary, based on the fact that only one-fifth of the total ovarian mass was analyzed [5]. This correction factor was used again by Hirshfield and co-workers in studies published 19 years later, which evaluated the effects of chronically elevated luteinizing hormone on the primordial follicle pool in mice after sampling every fifth ovarian section [6]. However, in 2001, results from a study co-authored by Hirshfield of the effects of ectopic Bcl-2 expression on the primordial follicle endowment in the rodent, ovary used a correction factor of 80 after sampling every tenth ovarian section [7]. The rationale for this was that "the number of primordial, primary, or preantral/antral follicles present in the marked sections was multiplied by 10 to account for the fact that every tenth section was used in the analysis and by 8 to account for section thickness" [7]. This type of correction factor has also been used routinely by others [e.g., [10–12]] and us [e.g., [13–21]] in studies of oocyte and follicle develop-

ment in the mouse ovary. Thus, which correction factor should be applied?

Intuitively, section thickness would seem to be an important variable. For example, if one serially sections a day 4 postpartum mouse ovary, which averages 400 μm in length ($n = 25$ mice), in 6- versus 8- μm widths, there will be approximately 67 6- μm sections and 50 8- μm sections. Irrespective of section thickness, however, only one-fifth of the ovary will be sampled in both cases, and thus multiplying all values by 5 to account for the remainder of the ovary not sampled should provide a reasonable estimate of absolute follicle numbers. However, if one counts every fifth section, ovaries sectioned at 6- μm widths would have approximately 3 more sections included in the counting process than ovaries sectioned at 8- μm widths. This suggests that an ovary sectioned at 6- μm widths will probably have more follicles tallied than the same ovary sectioned at 8- μm widths. Therefore, accounting only for that proportion of the ovary not included in the sampling analysis, while ignoring the effect of section thickness, does not completely address the issue of obtaining a realistic estimate of absolute follicle numbers. On the other hand, multiplying the values by section thickness probably gives spuriously high numbers, leaving open the question of how to accurately account for the effect of section thickness.

Whatever the case, a much more fundamental question remains. Does the application of a correction factor, regardless of whether it is 5 or 80, change the final conclusions of any study published to date? Absolutely not, as long as the correction factor was applied uniformly to all of the values used to produce the final results for analysis. Indeed, in a study of the relationship between ovarian innervation and folliculogenesis in the rat ovary, Malamed and colleagues estimated follicle numbers by determining the mean total number of follicles per section after sampling every fifth or sixth section [22]. This mean value was then multiplied by the total number of sections of the ovary to obtain an estimate of the total number of follicles per ovary. The authors state that "values obtained by this method tend to be spuriously high because the thickness of each section (5 μm) is about one third the mean diameter of a primordial follicle" [22]. Importantly, however, the authors point out that "regardless of the validity of the estimated absolute values for numbers of follicles per ovary, the uniform application of the method of calculation to the data from ovaries of all ages examined should produce valid relative values" [22]. This is a key point since in all studies cited herein that used a correction factor, the application of the correction factor was uniform. Therefore, the purpose of this commentary is not to call into question the validity of conclusions drawn from past studies of follicle numbers in the rodent ovary by any investigators,

but rather to explore the basis of the variance in follicle numbers per ovary reported by different laboratories.

It should be noted that there are also many studies that have used the sampling procedure to estimate follicle numbers in mouse or rat ovaries without the application of any correction factor prior to data presentation [e.g., [23–26]]. The conclusions drawn from these studies are no more or less valid than conclusions drawn from studies that have utilized a correction factor, despite the fact that the absolute values for follicle numbers are quite different among the various reports. Moreover, other types of follicle counting procedures have been reported for studies of the mouse ovary. These include counting every primordial follicle, as well as every growing follicle with the oocyte nucleus clearly visible, in every second section [27], to more intensive procedures involving the use of germ cell-specific markers with correction factors for oocyte and ovarian volume [3]. For example, in work published by Pepling and Spradling [3], the volume of each ovary analyzed was measured. A representative section from the ovary was then immunostained for the germ cell-specific protein, Vasa, and the number of Vasa-labeled cells in this section was counted. The authors then stated that "using the average diameter of a germ cell, the fraction of the ovarian volume represented by the counted section was then calculated. This allowed the number of germ cells in the whole ovary to be computed" [3].

With this information in mind, what assistance will this commentary be to the field of ovarian biology? If one thinks only in terms of the final conclusions drawn from the various studies of follicle endowment and depletion, the answer to this question is 'none' since the validity of these conclusions are not in question. However, if one thinks in terms of the absolute values for follicle numbers per ovary reported by different laboratories, a discussion of the advantages and drawbacks of the various approaches used to assess follicle numbers may begin to settle an emerging controversy. At least for our research group, this commentary has forced us to critically evaluate the correction factor we have employed in past studies of the post-natal mouse ovary [e.g., [13–21]], which attempted to account for both the fraction of the ovary sampled ($\times 5$, for every fifth section) and section thickness ($\times 8$, for 8 μm). Indeed, as indicated in the papers by Canning *et al.* [4] and Takai *et al.* [28], we now believe that when sampling every fifth section a correction factor of 5, rather than 40, provides a more reasonable estimate of absolute follicle numbers per ovary. Will this change anything? We believe it will, if one considers the following example. Using a germ cell counting procedure discussed earlier, Pepling and Spradling recently estimated that CD-1 female mice possess approximately 2,400 oocytes in their ovaries 4 days after birth [3]. We recently estimated that CD-1 fe-

males possess approximately 5,000 oocytes (follicles) at day 4 postpartum [4], using the every fifth 8- μm section sampling procedure with a correction factor of 5. Although there still remains a discrepancy in the absolute number of follicles per ovary between the two studies, a 2-fold difference is a vast improvement over the 16-fold difference that would have occurred if a correction factor of 40 rather than 5 were used.

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The references cited herein represent only a selection of the many published studies describing the effects of experimental agents or genetic manipulations on follicle numbers in laboratory animal models, and the author apologizes for not citing work from other laboratories owing to space constraints.

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