A new model of reproductive aging: the decline in ovarian non-growing follicle number from birth to menopause

Karl R. Hansen1,5, Nicholas S. Knowlton2, Angela C. Thyer3, Jay S. Charleston4, Michael R. Soules3 and Nancy A. Klein3

1Division of Reproductive Endocrinology and Infertility, Department of Obstetrics and Gynecology, University of Oklahoma Health Sciences Center, PO Box 26901, Oklahoma City, OK 73190, USA; 2NSK Statistical Solutions LLC, Choctaw, OK, USA; 3Seattle Reproductive Medicine, Seattle, WA 98109, USA; 4Stereotome NW, Issaquah, WA 98027, USA
5Correspondence address. Tel: +1-405-271-8722; Fax: +1-405-271-1655; E-mail: karl-hansen@ouhsc.edu

BACKGROUND: The primary determinant of reproductive age in women is the number of ovarian non-growing (primordial, intermediate and primary) follicles (NGFs). To better characterize the decline in NGF number associated with aging, we have employed modern stereology techniques to determine NGF number in women from birth to menopause. METHODS: Normal human ovaries were collected from 122 women (aged 0–51 years) undergoing elective oophorectomy, organ donation or autopsy. After gross pathologic examination, systematic random sampling was utilized to obtain tissue for analysis by the fractionator/optical disector method. Models to describe the resulting decay curve were constructed and evaluated. RESULTS: NGF decay was best described by a simple power function: log (y) = axb + c, where a, b and c are constants and y = NGF count at age x (R2 = 0.84, Sums of Squares Error = 28.18 on 119 degrees of freedom). This model implies that follicles decay faster with increasing age. CONCLUSIONS: Unlike previous models of ovarian follicle depletion, our model predicts no sudden change in decay rate, but rather a constantly increasing rate. The model not only agrees well with observed ages of menopause in women, but also is more biologically plausible than previous models. Although the model represents a significant improvement compared with earlier attempts, a considerable percentage of the variation in NGF number between women cannot be explained by age alone.

Keywords: reproductive aging; human ovary; optical fractionator; disector; stereology

Introduction

Reproductive aging in women is a continuous process that begins prior to birth and extends through the menopausal transition. The primary mechanism behind this process is the depletion of the ovarian pool of non-growing follicles (NGFs). Compared with other major organ systems, the human female reproductive system ages to the point of failure at a relatively young age (average 51 ± 8 years). Although menopause itself is an easily recognized end-point of the reproductive lifespan, dysfunction as a result of reproductive aging occurs many years prior to this event. Peak fertility occurs in the mid-twenties, after which there is a general decline in fertility with a steep decline beginning after age 35 (Menken et al., 1986; van Noord-Zaadstra et al., 1991). This observed decline in fertility occurs in spite of normal hormone secretion from the ovaries of ‘older’ reproductive-aged women, which is maintained until 3–4 years prior to menopause (menopausal transition) (Klein et al., 1996a, b; Burger et al., 1998; van Zonneveld et al., 2003; Hansen et al., 2005).

The wide age range at which natural menopause occurs suggests that there is considerable variation between women in the reproductive aging process. It is currently unclear whether this biological variation is due to differences in the initial endowment of NGFs, or alternatively, due to differences in the rate of NGF depletion. In spite of the considerable variation in the age at which ovarian dysfunction and failure occur, the sequence of terminal events is fairly predictable. Initially, menstrual cycle length shortens due to early follicular development and ovulation (Klein et al., 2002), followed by disruption of regular menstrual cyclicity (perimenopause), and finally, complete ovarian failure (menopause). The perimenopause is an overt indication that the number of remaining ovarian NGFs has dropped below a critical threshold (Richardson et al., 1987). Clinically, it is well accepted that once a woman becomes perimenopausal (oligomenorrhea in association with elevations in gonadotropins), her fertility is severely compromised (Santoro et al., 1996). At this point in the reproductive aging process, treatment
options are limited due to ovarian resistance to exogenous gonadotropins and aneuploidy of most remaining oocytes.

Given the marked variation in the reproductive aging process in women and the relative lack of longitudinal studies describing the progressive loss of ovarian reserve, there is considerable interest in developing a model that better describes NGF depletion. Such a model could be an important tool for forecasting the reproductive lifespan and predicting future fertility. Particularly, such a model would be useful in modern society, wherein many women are delaying childbearing for socioeconomic reasons. Furthermore, accurate models of reproductive aging would be relevant for women with a personal history of exposure to radiation, chemotherapy or prior ovarian surgery. Models to describe ovarian NGF depletion have been attempted (Faddy and Gosden, 1995, 1996; Faddy et al., 1992; Gougeon et al., 1994), but unfortunately these investigations have been subject to methodological issues that may limit the accuracy of the NGF estimates, and thus the resulting models.

Previous investigations have suggested that the initial NGF endowment in women is ~500 000–1 000 000 total follicles at birth (Forabosco et al., 1991; Faddy et al., 1992). Through a combination of recruitment toward dominant follicle development and atresia or ovulation, the stock of NGFs is depleted (Block, 1952; Gougeon and Chainy, 1987, 1994; Richardson et al., 1987; Faddy et al., 1992). When NGF numbers drop below a critical threshold (estimated to be ~1000–1100 follicles) ovulation ceases and the menopause ensues (Faddy et al., 1992). Mathematical modeling of NGF decay has suggested that the decay rate is exponential and biphasic, with an acceleration in the rate at the age of ~38 years when ~25 000 NGFs remain (Faddy et al., 1992). Realizing the biological implausibility of a sudden acceleration in follicular depletion, newer mathematical models have been proposed utilizing the same data in an attempt to ‘smooth’ the regression curve (Faddy and Gosden, 1996; Faddy, 2000). Nevertheless, these newer models either still contain two different rates of decay (Faddy and Gosden, 1996), or include ‘constants’ that are continuously changing (Faddy, 2000). Despite this limited data base and much conjecture from models, these numbers and concepts continue to be represented in the scientific literature as established facts.

The limitations of the models describing NGF decay may have their origins in the underlying data used to generate the models. In that regard, it is important to realize that the data used to generate the current models is based on combined data compiled from four independent investigations (Block, 1952, n = 43; Block, 1953, n = 6; Richardson et al., 1987, n = 9 and Faddy et al., 1992, n = 52). Although these studies used similar tissue processing and counting techniques, they were not identical. Furthermore, there was no attempt at inter-observer validation between investigations. Additionally, all of the above studies used ‘model-based’ techniques to derive the estimated NGF number within a given ovary. Model-based techniques are volume weighted, meaning that larger objects (follicles) appear in more sections and tend to be overcounted, whereas smaller objects appear in fewer sections and therefore are undercounted (Gundersen et al., 1988; West, 1993; Myers et al., 2004; reviewed in Charleston, 2000).

Model-based methods may or may not account for tissue section thickness and/or particle size, and generally incorporate correction factors which may or may not improve the accuracy of a given estimate. Within a given study these issues may not matter (trends from within a study may be valid due to consistency of bias); however, when combining results of different investigations these limitations become problematic.

We have recently described the use of modern stereology techniques to determine ovarian follicle number in non-human primates (Miller et al., 1997, 1999) and in women (Charleston et al., 2007). These design-based methods use a combination of the fractionator technique with a physical (Brændgaard and Gundersen, 1986; Miller et al., 1997) or optical disector (Gundersen et al., 1988; Myers et al., 2004; Charleston et al., 2007). The advantage of the fractionator/disector technique is that it avoids the introduction of correction factors that are necessary with older methods. Modern stereology methods using the fractionator/disector technique have become the standard in biostructural analysis, and have been utilized to estimate NGF number in the neonatal (Sonne-Hansen et al., 2003) and adult mouse ovary (Myers et al., 2004), nephron and glomerular number in the kidney (Bertram, 1995, 2001), germ cell number in the testis (Wreford, 1995; Kumar et al., 2001) and neuron number in the central nervous system (Brændgaard et al., 1990; West et al., 1991, Galvin and Oorschot, 2003; among others).

Given the limitations of previous models of NGF decay, and an understanding of the importance of developing models that more accurately predict reproductive age, the purpose of this investigation was to better characterize the decline in human ovarian NGF number associated with aging. To address these questions, we first determined ovarian NGF number in ovaries from 122 subjects, aged 0–51 years, using modern stereology techniques. Second, we constructed and evaluated models to describe the resulting decay curve.

Methods and Materials

Source of human ovaries

Normal human ovaries were obtained from women scheduled for elective unilateral or bilateral oophorectomy (n = 45) or through collaboration with organ donation agencies and a national tissue bank (see section acknowledgments, n = 77) from 2001–2006. Written informed consent was obtained from women scheduled for elective oophorectomy before collection of specimens or obtaining their medical history. Human subjects approved consent was obtained from a responsible family member of the deceased in all instances. Medical records, surgical pathology reports and operative reports were subsequently reviewed. Subjects previously exposed to chemotherapy, radiation or with previous ovarian surgery were excluded from participation. Specimens with gross or microscopic evidence of ovarian pathology or with ovarian cysts >2 cm diameter were also excluded from analysis. Human subjects committee approval for this investigation was obtained from both the University of Oklahoma (n = 29) and the University of Washington (n = 93).

Tissue preparation and follicle counting

Tissue preparation, follicle identification and counting were performed as previously described in detail by Charleston et al. (2007).
Briefly, ovaries were obtained as soon as practical following surgical removal or autopsy. In the case of elective surgical removal, a small sample of the ovary was removed and prepared separately for review by a surgical pathologist. The remainder of the ovary was then re-weighed. The difference in weights recorded was used to determine the fraction of the whole ovary that was available for use in this study (see fractionator sampling below). The ovary was then immersed in Bouin’s fixative (picric acid 0.9% w/v, formaldehyde 9% v/v and acetic acid 5% w/v) prior to additional preparation for stereology examination. After receiving whole or biopsied ovaries in fixative from the procurement source, one of the investigators carefully examined the tissue before it was deemed suitable as a study specimen. Specimens were rejected if they contained cysts >2 cm, areas of missing cortex, any obvious gross pathology, or were subsequently determined to have pathology on microscopic examination.

**Stereology**

The fractionator/optical disector method is based on directly counting the particles of interest (in this case, the oocyte nucleoli of NGFs) in a known fraction of the original structure. The total number of nucleoli encountered in this fraction is then multiplied by the inverse of a hierarchy of systematic random sampling fractions in order to generate an estimate of the total number in the original specimen (reviewed in Charleston, 2000).

For surgical specimens, the first sample fraction (F1) consisted of the original ovary minus the small portion previously removed for pathological examination. For both whole ovaries and post-surgical specimens, each ovary was cut into ~1 mm slabs perpendicular to the long axis of the ovary. Approximately eight slabs were selected out of the total generated (yielding a second fraction, F2) using systematic random sampling rules. The selected slabs were dehydrated in a graded ethanol series, passed through a transition solvent of 100% acetone, and embedded as a group in one or two large (2’ × 3’) blocks of glycol methacrylate, a plastic-like substance (GMA, Technovit 8100, Energy Beam Sciences, Inc., Agawam, MA, USA) as described by Charleston et al. (2003). The blocks were exhaustively sectioned at a thickness of 25 μm using a rotary microtome. Every 10th section (the third fraction, F3) was collected in the order generated on glass slides for staining. Sections were stained with Richardson’s stain as previously described (Miller et al., 1999), and then mounted with coverslips using Cytoseal 280 (Stephens Scientific, Kalamazoo, MI, USA).

Sections representing the largest two-dimensional profile of each slab were then selected for counting with the optical disector. The fraction that this section represented from the entire collected stack of sections from each slab (F4) was determined by placing a point grid over the section and summing the points that fell over the sections. This value was then divided by the total number of points landing over all collected sections (including the initial and trailing partial slab fragments encountered at the beginning and end of the sectioning run across each slab).

Optical disector counting frames (three-dimensional cubes) were placed over the selected stained sections using systematic random sampling rules (Gunderson et al., 1988). Placement of optical dissectors and delineation of the areas of interest was accomplished by use of Stereoinvestigator software (MicroBright Field, Colchester, MA, USA) operating on a PC style computer coupled to a Nikon (University of Oklahoma) or Zeiss Photomicroscope II (University of Washington). Sequential placement of optical disector frames was performed by a motor driven microscope stage directed by the Stereoinvestigator software.

The entire cortex of each section in the counting sample was outlined under low magnification for placement of the disector frames. The area of the disector frame divided by the area of the steps between placements (representing a grid) represented a fifth sampling fraction (F5). The next sampling fraction (F6) consisted of the height of the optical disector divided by the height of the tissue section. This fraction accounts for the portion of the tissue section represented by the guard area, in which no counting was performed.

**Follicle identification**

NGFs were counted when a clearly defined oocyte nucleus was present within the optical disector counting frame (Charleston et al., 2007). All follicles were classified according to the morphologic criteria as described by Gougeon (1996). The population of NGFs consisted of primordial, intermediate and primary follicles (Fig. 1). Primordial follicles were defined as containing a single layer of flattened granulosa cells; intermediate follicles were defined as a single layer of granulosa cells with at least one cuboidal and one flattened granulosa cell; and primary follicles were defined as containing a single layer of cuboidal granulosa cells without any flattened granulosa cells (Fig. 1). Raw counts (Q̃) for each class of NGFs were then converted to an estimate of the total number (N) of NGFs in the entire ovary by the following equation (where ‘Q̃’, number of each class of NGF identified in the fraction of tissue counted):

$$N = \sum Q̃ \times \frac{1}{(F1 \times F2 \times F3 \times F4 \times F5 \times F6)}$$

Analysis of the precision of NGF counts was determined at the level of the individual ovary by calculating the observed coefficient of error (OCE) as described by West and Gundersen (1990).

**Statistical analysis and modeling**

Both ovaries within a pair were counted for the first 48 subjects. Three observations led us to conclude that a single, rather than both ovaries within a pair, could be counted to obtain a reasonable estimate of the total NGF endowment of an individual. First, a pair analysis comparing the number of NGFs in one ovary versus the other demonstrated no significant difference (Wilcoxon signed rank test, P = 0.81). Second, there was a direct correlation in the number of NGFs between the two ovaries from an individual (Spearman rank correlation = 0.92, P < 0.0001). Finally, the median variation of 32% in NGF number between ovaries within a pair (Charleston et al., 2007) was not significantly different than the variability in estimated counts observed in recount experiments of the same ovary (15–29%, with greater variation at lower follicle number, Charleston et al., 2007). As a result, only a single ovary within a pair was counted for the remaining 74 subjects. As most investigators consider primordial, intermediate and primary follicles to be ‘NGF’, and thus represent ovarian reserve, our data is presented in terms of the NGF number. An estimate of total NGF number for each subject was determined by summing NGF number for both ovaries from subjects in which both ovaries were counted. For subjects in which a single ovary was counted, the NGF number was multiplied by two. NGF number was log-transformed and plotted versus chronological age. Models to describe NGF decay were constructed and evaluated with Matlab R13 software (Natick, MA, USA). The goodness-of-fit of the different models was determined by calculating the $R^2$ and the Sums or Squares Error (SSE) values for each model. The larger the $R^2$ and the smaller the SSE values the better the given model describes NGF decline with advancing chronological age. Only models with different degrees of freedom (DF) can be statistically compared to the others via a difference in the SSE. When appropriate and possible, models were compared using the chi-square distribution. A value of $P < 0.05$ was considered significant.
Results

One-hundred and twenty-two subjects were enrolled in the investigation and had one or both ovaries processed for determination of total ovarian NGF count. The average number of NGFs was 130,286 $\pm$ 18,980 (mean $\pm$ SEM), with a range of 0–916,500 NGFs (Table I). The average OCE (a measure of precision for the individual NGF count estimates) was 0.18 $\pm$ 0.01. The average ovarian NGF counts for different age groups are illustrated in Fig. 2. NGF counts were log-transformed and plotted versus chronological age for model construction and evaluation.

First, a linear model of human ovarian NGF decay was analyzed (Fig. 3a). This model predicts an estimated number of 3,834,864 NGF at birth. In general, the linear model is a poor fit to the data ($R^2 = 0.50$, SSE = 89.30, on 120 DF), estimating that NGFs would not be depleted until on average, age 59. Furthermore, at the average age of menopause (51 years), the model predicts 2,290 NGFs remain.

Next, an exponential model was evaluated (illustrated in Fig. 3b). This model appears to be an even poorer fit to the data, based on what appears to be a decline in the rate of follicle decay with advancing age. Only 46% of the decline in NGF number with advancing age is described by the model ($R^2 = 0.46$, SSE = 95.47, on 120 DF). Additionally, the model predicts 2,754,228 NGF at birth and 3,881 NGFs remaining at the average age of menopause. The $R^2$ value cannot be calculated for this model, as it is really a combination of two models; however, the SSE value of 81.23 (118 DF) demonstrates that the biphasic-exponential model is a better fit than either the linear or exponential models ($P < 0.05$ in both cases).

In the final analysis, NGF count decay was best described by a simple power function: $y = ax^b + c$, where $a$, $b$ and $c$ are constants and $y = $ NGF count at age $= x$ (Fig. 4). This model implies that follicles decay faster with increasing age. The power function model gave a robust fit ($R^2 = 0.84$, SSE = 28.18 on 119 DF) to the observed data, with age alone accounting for 84% of the variability in NGF count. Comparing the power model to the linear, exponential and biphasic-exponential models, the power model resulted in a statistically improved fit to the data ($P < 0.001$) in all cases. Finally, we also evaluated the fit of Faddy’s differential equation model (Faddy, 2000) to our data. The power model was a better fit to the data than the Faddy differential equation model ($R^2 = 0.84$, SSE = 28.18, 119 DF, versus $R^2 = 0.79$, SSE = 37.0, 119 DF, power model versus Faddy model, respectively) based on the $R^2$ and SSE values; however, direct statistical comparisons of the two models are not possible due to both models having the same DF. The power model predicts the total NGF count

Figure 1: Follicle distribution and identification in the human ovary
Illustration of NGFs and antral follicles in the ovary of a ‘younger’ (a) and ‘older’ (b) reproductive aged woman. NGFs consist of primordial (c, bar = 20 $\mu$m), intermediate (d, bar = 20 $\mu$m) and primary (e, bar = 20 $\mu$m) follicles. This pool of NGFs is generally believed to be representative of the ‘ovarian reserve’. Once the theca interna differentiates in the pre-antral follicle, it becomes gonadotropin responsive and the follicle is considered to have entered the growth phase. Antral follicles (f, bar = 50 $\mu$m) represent a more advanced stage of follicular development. A cohort of antal follicles is recruited in the early follicular phase in response to an elevation in gonadotropin levels. In most cycles, a single follicle ovulates and the majority of the cohort undergoes atresia. The number of large antral follicles (2–10 mm) visible on transvaginal ultrasound examination is also correlated with chronological age.

702
at birth is 521 194. Setting the average age of menopause at 51 years, our model predicts 758 NGFs remain in both ovaries at birth is 521 194. Setting the average age of menopause at 51 years, our model predicts 758 NGFs remain in both ovaries at 24 months, with 95% confidence interval for the age of menopause between 41.9 and 57.8 years. This predicted interval agrees well with large cohort studies investigating the natural age of menopause (Treloar, 1981). The resulting model that best fits all of the biological parameters is:

\[
\log(\text{NGF count}) = (-0.00019) \times (\text{age in years})^{2.452} + 5.717.
\]
When the analysis was restricted to primordial follicles only, as opposed to total NGF number, the power model as described above is still a better fit to the data than any of the other models. Given that our estimate of total NGF endowment for subjects was estimated by summing both ovaries within a pair for subjects that had both ovaries counted, and doubling the count for subjects with a single ovary counted, we also evaluated the goodness-of-fit of the power model to NGF number when the analysis was restricted to a single ovary. Whether the analysis is restricted to the ‘high-count’ or the ‘low-count’ ovary in those individuals with both ovaries counted, the power model is still the best fit to the data (data not shown).

We also evaluated the goodness-of-fit of the power model to ovarian NGF numbers obtained in previous investigations [43 subjects from Gougeon et al. (1994), 13 subjects from Richardson et al. (1987) and 50 ovaries from Block’s (1952,1953) investigations]. The power model was a better fit to the data than the previously described biphasic-exponential model ($R^2 = 0.83$, SSE 19.71 on 103 DF versus SSE 25.25 on 102 DF, $P < 0.05$), with an estimated 510 505 NGFs at birth and 1174 remaining at the average age of menopause. Furthermore, the power model was also a better fit than the Faddy (2000) model ($R^2 = 0.83$, SSE = 19.71, 103 DF, versus $R^2 = 0.78$, SSE = 24.9, 103 DF, power model versus Faddy model, respectively).

**Discussion**

In this manuscript, we present a power model which describes the decline in human ovarian NGF number associated with aging. Unlike previous models (e.g. a biphasic-exponential model), the power model suggests that the decay of NGFs is constantly accelerating rather than suddenly increasing at ~38 years. It suggests that age accounts for ~84% of the variation in NGF count at different ages, and agrees well with the known distribution of menopausal age. Strengths of our new model of reproductive aging include that it was developed with the largest number of subjects ever studied ($n = 122$) and spans the entire age range from birth to menopause. Furthermore, all of the NGF estimates were obtained by a single group of investigators using the same modern counting technique.

Our investigation into the decline in NGF number associated with aging is the first to re-address this issue with entirely new data in many years. Relatively few investigations of this nature have been performed due the difficulty in obtaining appropriate tissue for evaluation and the laborious nature of tissue preparation and follicle counting. Block described ovarian follicle number in 10 newborns (Block, 1953) and 43 girls and adult women (age 6–44 years, Block 1952). Richardson et al. (1987) determined ovarian follicle number in 17 women, aged 45–55 years old. More recently, Gougeon determined ovarian follicle number in 52 women, aged 19–51 years (Faddy et al., 1992). The pioneering work of these investigators led to the understanding that ovarian follicle number decreases with increasing age, and that ultimately, few, if any follicles remain following menopause. Furthermore, Richardson’s (1987) investigation suggested that the rate of decline in NGF number accelerated with increasing age.

Using the combined data from these investigators, Faddy et al. (1992) first suggested that the decline in ovarian follicles associated with aging was best described by a biphasic-exponential model, which was a better fit to the data than either a linear or single exponential model. The model suggested that the total follicular endowment at birth was ~952 000, with an initial rate of decay of ~0.097. At the age of ~38 years and a follicle count of ~25 000, a sudden increase in decay occurred to over two-fold the initial rate (~0.237).

Limitations of these earlier investigations include the use of model-based techniques for estimations of ovarian follicle number, and ultimately the combination of data obtained with similar, but different techniques, to derive the models. Model-based methods as utilized in the previous investigations involved embedding the ovaries in paraffin followed by serial
sectioning and counting every 200th section (Block, 1952, 1953), the first 10 out of 100 sections (Richardson et al., 1987), and ‘up to one in 200’ sections (Faddy et al., 1992). The number of follicles counted was then multiplied by the sampling fraction (i.e. multiply the raw count \( \times 200 \) in the Block studies). In Block’s investigations, the resulting counts were then multiplied by a correction factor that took into account the section thickness and the diameter of the particle of interest as initially described by Floderus (1944) and Abercrombie (1946). These correction factors attempt to improve the precision of the estimates by taking into account the observation that larger particles (follicles) appear in more sections and tend to be over-counted, whereas smaller particles appear in fewer sections and tend to be undercounted. To what extent the incorporation of correction factors improves the accuracy of the estimate is unknown. Richardson et al. (1987) incorporated no such correction factor, and Faddy et al. (1992) incorporated a correction-factor ‘as appropriate’, although when and how it was applied is uncertain. Given the disparate techniques used to obtain the estimates in these early investigations, combining the estimates and deriving models from the combined data seems challenging at best.

The design-based methods combining the fractionator and optical disector techniques utilized in this investigation require no such correction factors. Additionally, they are relatively efficient compared with older techniques (Brændgaard et al., 1990; Charleston, 2000; Charleston et al., 2003, 2007). Finally, our investigation is not confounded by the use of different techniques, as the same method was utilized to obtain estimates of NGF number for all of the subjects in the study. Although we believe the fractionator/optical disector technique offers considerable advantages over older model-based techniques, it should be noted that our NGF estimates for women at different chronological ages were not significantly different from earlier estimates derived using model-based techniques with the exception of the 21–30-year-old age group, where the fractionator/optical disector technique yields higher estimated counts (data not shown). This high degree of concordance in NGF count estimates derived with the different methods demonstrates that model-based techniques can provide high-quality estimates of NGF count when meticulously applied as was done in previous investigations.
Our power model of NGF decay fits the data from previous investigations well, explaining 83% of the variability in NGF count with the older data. This is similar to our current dataset, in which 84% of the variability is explained by age. Given the improved fit of this model on the older dataset compared with the biphasic-exponential model, we feel that the power model was simply overlooked during previous modeling attempts.

Faddy et al. realized that the biphasic-exponential model with its abrupt change in the rate of follicle depletion was unrealistic, and has proposed two additional models to describe follicle decay (both using the same combined data as described above). The first modification (Faddy and Gosden, 1996) describes follicle decay with a stochastic threshold model, and the second (Faddy, 2000) a differential equation. Both modifications result in an improvement in the fit of the resulting curve to their data; however, both are more complicated than our model and neither fits our data (or the earlier dataset) better than a power model. Consider the power model of NGF decay versus the Faddy (2000) differential equation model: power model: \( \log(y) = ax^b + c \), where \( a \) and \( b \) and \( c \) are constants and \( y \) = NGF count at age \( x \).

Faddy model: \( dy/dx = -(\alpha + \beta(\gamma + y))y \), where \( dy/dx \) represents the ‘instantaneous rate of temporal change’, \( y \), ‘the number of follicles remaining at that time’, and \( \alpha \), \( \beta \) and \( \gamma \) ‘are parameters which result in a gradual increase in \( (\alpha + \beta)/(\gamma + y) \) from close to \( \alpha \), when the number of follicles present is very large, toward \( \alpha + (\beta/\gamma) \) when the number of follicles declines’.

Although the argument can be made that both models are similar in that they describe follicular decay as a single function, clearly the power model is simpler to the non-mathematician.

Several limitations of our investigation should be noted. First, many of our subjects (\( n = 45 \)) were having one or both ovaries removed surgically for a benign indication. Therefore, the argument could be made that our subjects were not ‘normal’, and may not be representative of the general population that had no such indication for oophorectomy. However, there were no significant differences in the NGF count in ovaries from women of similar ages that were removed at surgery or at the time of autopsy (data not shown), suggesting that this is unlikely. Furthermore, ovaries counted in Richardson’s study were also obtained at the time of surgical oophorectomy. These counts were included in the original models to describe follicle decay, and we believe it is unlikely that the inclusion of these individuals would have significantly altered our model.

Second, our model was constructed based on estimates of a subject’s total NGF endowment. We estimated this number by summing the NGF counts for both ovaries when both were counted, and doubling the count for a single ovary when only a single ovary was counted. It could be argued that the later calculation may not be an accurate estimate of total NGF endowment, as the coefficient of variation (CV) between ovaries within an individual averages 32% (Charleston et al., 2007). However, we feel this estimate is reasonable given that differences in NGF count between ovaries within a pair are not statistically greater than the observed differences in NGF count in recount experiments of the same ovary (15–29% CV, with greater variation at lower follicle numbers, Charleston et al., 2007). This relatively high CV in our recount experiments highlights the difficulty of investigations of this nature in human ovaries in general. Particularly at lower NGF counts, the relatively heterogeneous distribution of NGFs in the ovarian cortex makes any counting technique challenging. As a result, our new counts must also be considered estimates of NGF number. Nevertheless, we have avoided many of the biases associated with older techniques and the methodology for counting all of the ovaries in our investigation has been identical.

In considering the power model of NGF depletion, two questions become apparent: (i) what biological processes are responsible for the constantly accelerating rate of NGF depletion? and (ii) how useful is the resulting model in forecasting the reproductive lifespan for any particular woman?

The majority of ovarian follicle loss associated with aging is attributable to an increased rate of atresia, as the rate of loss due to ovulation remains relatively stable until a few years prior to menopause. Whether this increased rate of atresia is due to an increased rate of NGFs entering the growth phase as suggested by Gougeon et al. (1994) or due to atresia of NGFs prior to entering the growth phase (Faddy and Godsen, 1995) is debatable. With the former model, it is tempting to speculate that increasing serum concentrations of FSH as observed in older reproductive aged women (Klein et al., 1996b; van Zonneveld et al., 2003) could contribute to the acceleration of follicles leaving the resting phase to enter the growth phase. Ultimately, this higher rate of recruitment would result in a positive feedback cycle, wherein the rate of follicle depletion correlates with rising FSH levels. In the latter model, it is unclear what physiological mechanisms might be responsible for accelerated atresia of NGFs, although it is also possible that rising serum levels of FSH associated with aging may play a role in this regard. Previous investigations have suggested that follicle FSH receptor expression begins only at the primary stage, with earlier follicles (primordial and intermediate) having no such expression (Oktay et al., 1997; Rice et al., 2007). However, it is important to realize that previous investigations have included ovarian follicles from fewer than 20 total subjects, and no attempt has been made to stratify expression based on the age of the subjects. Furthermore, the expression of FSH receptors even at the primary follicle phase is not absolute, ranging from 21 to 33% (Oktay et al., 1997; Rice et al., 2007). These findings suggest that NGFs from women at different ages may have differing capabilities in terms of their response to physiological changes in hormone levels associated with aging.

With regards to the second question, although the power model represents a considerable improvement in our understanding of the reproductive aging process relative to prior modeling attempts and has utility from a population standpoint, it still has significant limitations. A full 16% of variation in ovarian NGF number is not explained by the model and, in its current form, it is inadequate for predicting the reproductive lifespan for an individual. For example, one 30-year-old subject in the study (number 35) had an estimated NGF count of 9405, while a second (number 36) had a count of 234 164. The first subject was within a few years of
menopause, whereas the second had many years of reproductive lifespan remaining. Nevertheless, an individual woman could certainly be made aware of the standard decay curve and the impact of declining NGF number on fertility. It is important to realize; however, that the only parameter in the current model of NGF loss is chronological age. Perhaps the incorporation of other variables such as clinical markers of ovarian reserve (e.g. early follicular phase FSH and antral follicle counts obtained by transvaginal ultrasound examination) and lifestyle factors would improve the predictive power of the model. We are currently investigating these possibilities.

With the development and application of modern and efficient techniques for determining ovarian NGF count, we now have the tools available to address these important questions.

Acknowledgements
The authors would like to thank Theresa Naluai-Cecchini and Julia Massey for research coordination efforts among sites and for counting ovaries, and Lynne Charleston for her efforts in modifying and adapting stereology techniques to the human ovary. We would also like to thank the Pathology Department at the Universities of Oklahoma and Washington for assistance with collection of ovaries, and the North-west Tissue Center, Life Center Northwest, and the National Disease Research Interchange for procurement of ovaries. Presented in part at the 2006 American Society for Reproductive Medicine annual meeting.

Funding
OCAST (HR04-115 to K.R.H.) and NIH (R29-HD37360-04 to N.A.K.).

References


Galvin KA, Oorchot DE. Continuous low-dose treatment with brain-derived neurotrophic factor or neurotrophin-3 protects striatal medium spiny neurons from mild neonatal hypoxia/ischemia: a stereological study. *Neuroscience* 2003;118:1023–1032.


Kumar TR, Varani S, Wreford NG, Telfer NM, de Krester DM, Matzuk MM. Male reproductive phenotypes in double mutant mice lacking both FSH beta and activin receptor IIA. *Endocrinology* 2001;142:3512–3518.


Submitted on August 23, 2007; resubmitted on November 16, 2007; accepted on December 4, 2007.