

STP Position Paper: Ovarian Follicular Counting in the Assessment of Rodent Reproductive Toxicity

STP OVARY EVALUATION WORKING GROUP

KAREN S. REGAN,¹ (CHAIR), J. MARK CLINE,² DIANNE CREASY,³ BARBARA DAVIS,⁴ GEORGE L. FOLEY,⁵
LYNDA LANNING,⁶ JOHN R. LATENDRESSE,⁷ SUSAN MAKRIS,⁸ DANIEL MORTON,⁵ SABINE REHM,⁹
AND KENNETH STEBBINS¹⁰

¹Regan Path/Tox Services, Inc, ²Wake Forest University School of Medicine, ³Huntingdon Life Sciences, ⁴NIH/NIEHS, ⁵Pfizer,
⁶Otsuka Maryland Research Institute, ⁷NCTR, ⁸EPA/OPPTS (liaison), ⁹GlaxoSmithKline, ¹⁰The Dow Chemical Company

INTRODUCTION

Evaluation of the ovary is an important endpoint in toxicological assessments because xenobiotics that cause loss of oogonia, oocytes, or supportive somatic cells may have adverse effects on reproduction. To make an adequate histologic assessment of the ovary, knowledge of all morphologic components and an understanding of the changes that occur during the normal estrous cycle and aging are required. A thorough, qualitative light microscopic examination by a toxicologic pathologist will detect morphologic features associated with most functional alterations, including changes in the relative number or density of ovarian components. Development of consistent, reliable, and cost-effective quantitative methods to evaluate ovarian toxicity has been challenging. Much effort has been spent developing adequate methods for quantification of small follicles. The publications by Bolon et al. (1997) and Bucci et al. (1997) provide the most thorough recent comparisons of follicle-counting techniques in mice. These follicular-counting methods have been adopted and/or modified for use as first-tier screening methods in some regulatory guidelines for reproductive toxicity studies.

Regulatory Guidance for Reproductive Toxicity Studies

Current guidelines for microscopic examination of reproductive organs in reproductive toxicity studies are summarized in Table 1. The U.S. Environmental Protection Agency (EPA), U.S. Food and Drug Administration (FDA), and the Organization for Economic Cooperation and Development (OECD) guidelines for 2 generation reproductive studies recommend qualitative and quantitative evaluation of primordial follicles in the ovary [U.S. EPA, 1998; U.S. FDA (food additives) 2000; OECD, 2001]. The number of animals, number of sections, and selection of sections within the ovary are determined by the sponsor, but must be adequate for statistical analysis. In the EPA and OECD guidelines, quantification is restricted to ovaries of the F1 generation, but the FDA guideline for food additives (not pharmaceuticals) recommends follicle counts for both generations. The FDA guideline also recommends a qualitative assessment of the presence

or absence of growing follicles and corpora lutea. Regulatory guidelines for general toxicity studies do not include ovarian follicle counting.

Qualitative Assessment of the Ovary

Detection of female reproductive toxicity in reproductive toxicity studies relies on a combination of methods, including evaluation of estrous cycle duration, fertility assessments, enumeration of corpora lutea at necropsy, organ weights, and histopathology. The use of multiple assessment techniques helps to ensure a thorough evaluation that minimizes the risk of missing a true reproductive toxicant. The first step in the histopathologic assessment of the ovary is a thorough qualitative histologic examination using sections stained with routine stains, such as hematoxylin and eosin. The histology and physiology of the ovary necessary to make a thorough qualitative assessment have been reviewed (Manson and Mattson, 1992; Peluso, 1992; Peluso and Gordon, 1992; Davis et al., 1999; Yuan and Foley, 2002). Immunohistochemical procedures also have been developed that label small follicles and make them easier to detect, such as immunohistochemistry for nuclear CYP1B1 or PCNA in oocytes (Muskhelishvili et al., 2002).

Qualitative microscopic examination is an excellent first-tier screening tool for reproductive toxicity. With continuous dosing, ovarian toxicity may occur *in utero*, between birth and puberty or in adulthood. These exposure scenarios and the fact that ovaries are routinely examined only in adulthood in 2-generation reproductive studies contribute to the variety of patterns and degrees of severity of lesions observed in small follicles. Ongoing apoptosis or necrosis and decreases in the reserve pool of small follicles usually are detectable by qualitative microscopic examination. Degeneration of oogonia *in utero* or in the immediate postnatal period may cause significant depletion of primordial follicles that would likely be further exacerbated in adults due to continued recruitment for growth from the already depleted primordial pool (Mandl and Zuckerman, 1952; Krarup, 1969; Murphy and Beamer, 1973; Hirshfield, 1994). These changes are discernible to a pathologist conducting a focused qualitative examination of the ovary. If the loss of primordial follicles is complete or nearly complete, ovarian atrophy (characterized by an absence of follicles in all phases of maturation)

Address correspondence to: James Klaunig, Editor-in-Chief, Toxicologic Pathology Editorial Office, 199 Grassmur Turn, Pine Hill, NJ 08021.

TABLE 1.—Regulatory guidance for examination of female reproductive tissues in reproductive/fertility studies.

Guideline	Organs to be weighed	Organs to be collected/examined	Special procedures
Pharmaceuticals ICH S5A Detection of toxicity to reproduction for medicinal products (1994) and ICH S5B maintenance of the ICH guideline on toxicity to male fertility: An addendum to the guideline on detection of toxicity to reproduction for medicinal products (1996)		Ovaries and uterus for possible examination and evaluation on a case-by-case basis	
Industrial and agrochemicals OECD 416 2 generation reproductive toxicity study (2001).	Uterus, ovaries	Uterus with cervix, ovaries, vagina	Weigh ovaries separately. Qualitative examination of ovaries from F0 generation to detect primordial follicle depletion. Quantitative evaluation of F1 ovaries to count number of primordial follicles (may be combined with small growing follicles). Number of animals, number of ovarian sections, and section selection procedure is optional but should be statistically valid.
Industrial and agrochemicals OECD 421 Reproduction/developmental toxicity screening test (1995)		Ovaries	
Industrial and agrochemicals U.S. EPA OPPTS 870.3800 reproduction and fertility effects (1998).	Ovaries, uterus (with oviducts and cervix)	Ovaries, uterus (with oviducts), cervix, vagina	Qualitative examination of ovaries from F0 generation to detect primordial follicle depletion. Quantitative evaluation of F1 ovaries to count number of primordial follicles (may be combined with small growing follicles). Number of animals, number of ovarian sections, and section selection procedure are optional but should be statistically valid.
Industrial and agrochemicals U.S. EPA OPPTS 870.3550 Reproduction/developmental toxicity screening test (2000).		Ovaries, uterus	Qualitative examination of ovaries should detect primordial follicle depletion.
Agrochemicals Japanese MAFF.* Requirements for safety evaluation of agricultural chemicals (Nov. 24, 2000). Notification 8147	Ovaries, uterus	All reproductive organs	
Food additives and components U.S. FDA Redbook 2000 IV.C.9.a Guidelines for reproduction studies (2000).	Ovaries, uterus	Ovaries with oviducts, uterus with cervix, vagina	Qualitative examination of ovaries to detect primordial follicle depletion. Quantitative evaluation of F0 and F1 control and high dose ovaries to count number of primordial follicles. Suggested procedure: take 5 sections at least 100 μ apart from the inner third of each ovary. Count total primordial follicles in all 10 sections. Examination should also confirm presence or absence of growing follicles and corpora lutea relative to control ovaries.

*Japanese Ministry of Agriculture, Forestry, and Fisheries.

as well as secondary atrophy of the uterus and vagina and changes in mammary tissue will be observed in the qualitative assessment.

Chemicals such as 4-vinylcyclohexene diepoxide and other congeners of 1,3-butadiene and structurally related chemicals have very specific effects on primordial and primary follicles. Although these lesions may not be readily apparent in acute studies, these and similar chemicals cause significant ovarian atrophy and ovarian tumors in chronic bioassays (Morrissey et al., 1990; Hoyer and Sipes, 1996). Such potent carcinogens and mutagens also are associated with male reproductive toxicity and developmental toxicity (Morrissey et al., 1990). Importantly, effects on primary follicles are observed as increased atresia that is readily detected by qualitative morphological assessment. Similarly, cyclophosphamide, dimethylbenz(a)anthracene (DMBA), and many other chemicals known to target primordial follicles also affect primary follicles (Mattison, 1983; Mattison et al., 1983; Davis and Heindel, 1999). Such chemicals are also considered to be developmental and/or testicular toxicants because

of their mutagenic and cytotoxic activities. Thus, most if not all ovarian toxicants leave their fingerprints for discovery in careful qualitative histological evaluations. Moreover, most chemicals that cause significant decreases in follicle counts were identified as female reproductive toxicants by other assays. Indeed, in the proof of principle studies, the evaluations of the utility of follicle counts were based on female reproductive toxicants previously recognized by standard means that did not include follicle counting (Mattison, 1983; Bolon et al., 1997; Heindel, 1999). There is little evidence to suggest that ovarian toxicants would be overlooked during careful qualitative microscopic assessment.

Origins of Ovarian Follicular Counting

Prior to 1998, prenatal developmental toxicity (teratology) study guidelines included the counting of corpora lutea by gross inspection of the ovary to provide correlation with the number of uterine implantation sites. However, studies in mice and rats showed enumeration of follicles to be a sensitive means of estimating the extent of ovarian toxicity in females

exposed to xenobiotics (Toaff et al., 1979; Mattison et al., 1983; Takizawa et al., 1984; Weitzman et al., 1992; Flaws et al., 1994; Perez et al., 1997). The feasibility of using follicle counts as a screen for chemically induced ovarian toxicity in mice was assessed during Reproductive Assessment by Continuous Breeding (RACB) bioassays in the National Toxicology Program (Bolon et al., 1997). Data from these studies suggested that differential follicle counts can provide a quantifiable endpoint of ovarian injury in conventional bioassays, and in some instances, may provide a more sensitive indicator of female reproductive toxicity than fertility assessments in rodents.

In 1998, these findings led to the inclusion of ovarian follicle counts in the guidelines for reproductive toxicity studies (U.S. EPA, 1998) and subsequently, in the FDA reproductive toxicity guideline for food additives (U.S. FDA, 2000) and the Organization for Economic Cooperation and Development (OECD) guideline for fertility studies (OECD, 2001). Using data from the Bolon and Bucci reports, further discussion of oocyte quantification and ovarian histology was presented in a review article that was prepared for an International Life Sciences Institute (ILSI) workshop on reproductive endpoints for human health risk assessment (Heindel, 1999). Detailed methods of sectioning and counting are given elsewhere (Smith et al., 1991; Bolon et al., 1997; Bucci et al., 1997; Heindel, 1999). Most published reports of follicle-counting methods, however, describe studies in the mouse, and it is uncertain if the methods developed in mice reflect best practices for rats or identify hazards not detected by other standard methods used in risk assessment.

Quantitative Assessment of Small Follicles in Assessing Ovarian Toxicity

Properly conducted follicle counts can supplement qualitative ovarian assessment to characterize ovarian toxicants, understand their site of action, and assess primordial follicle integrity when ovarian lesions are subtle. The utility of follicle counts to detect female reproductive toxicity in rats that cannot be detected by other means has not been demonstrated adequately. Data submitted to regulatory agencies that may support the use of follicle counting for this purpose generally are not publicly available. Data generated from follicle counts can be highly variable with large standard deviations both between animals and between groups, making interpretation difficult. In part, this variability can be caused by sampling methods that are inadequate for consistent quantitative evaluation. As with other quantitative data, the methods used to obtain the sections and the quality of the sections evaluated are critical to the outcome. In addition to the high variability of the data, follicle counts can be extremely time-consuming. As a consequence, follicle counts may use up significant resources without detecting follicle damage and/or ovarian toxicity. When depletion or lesions of small follicles are clearly identified during a qualitative histologic examination, follicle counts may add little or no additional information for chemical hazard identification.

STP Recommendations

The evaluation of ovarian toxicity should use a weight-of-evidence approach, considering the qualitative histopathol-

ogy data and other available data (fertility data, clinical observations, organ weights, male reproductive toxicity data, etc.). The Society of Toxicologic Pathology recommends a 2-tier approach to evaluation of the rodent ovary in general toxicity or reproductive toxicity studies. During the first-tier assessment, ovarian sections should be evaluated qualitatively by light microscopy. The examination should be conducted by a toxicologic pathologist familiar with the normal reproductive cycle in the species, and should include evaluation of all major components of the ovary (follicles, corpora lutea, stroma, interstitium, and vasculature), with special attention given to the qualitative assessment of primordial and primary follicles. This qualitative assessment of ovaries should be done in conjunction with microscopic evaluation of the entire reproductive tract, and with consideration of all ancillary reproductive data (organ weights, estrous cyclicity, etc.).

Currently there is insufficient evidence to justify the use of follicle counting as a first-tier screening method in rodent toxicity studies. There is little published information supporting the ability of follicle counting to detect reproductive toxicity that could not be detected by qualitative microscopic assessment or other standard methods. If performed appropriately, quantification of small follicles may provide additional information as a second-tier procedure to further characterize suspected or demonstrated ovarian toxicants. The decision to perform a quantitative assessment of small follicles for purposes of risk assessment should be made on a case-by-case basis considering all existing data. Factors that might be considered when deciding whether or not to count small follicles include qualitative histological changes in the ovary such as increased atresia, changes in morphology or reduced numbers of corpora lutea or follicles; changes in ovarian weights; decreased fertility; changes in estrous cyclicity; the presence of testicular toxicity; evidence of developmental toxicity in offspring; and whether or not the addition of quantitative data may alter risk assessment. Tissue sectioning and follicle-counting methods developed in mice have not been adequately assessed and optimized in rats. A retrospective evaluation of follicle counts from rat studies, including those studies submitted to regulatory agencies, would be helpful in evaluating the methodology and utility of follicle counts in risk assessment. Additional studies are needed to determine and confirm a reliable standard method to prepare rat ovarian sections for quantitative assessment and to demonstrate the value of follicle counts in rats. As more information becomes available, the value of quantification of small follicles as a first-tier screening tool should be reassessed. Quantitative assessment of ovarian follicles should be performed only when it can improve risk assessment.

REFERENCES

- Bolon, B., Bucci, T. J., Warbritton, A. R., Chen, J. J., Mattison, D. R., and Heindel, J. J. (1997). Differential follicle counts as a screen for chemically induced ovarian toxicity in mice: results from continuous breeding bioassays. *Fund Appl Toxicol* **39**, 1–10.
- Bucci, T. J., Bolon, B., Warbritton, A. R., Chen, J. J., and Heindel, J. J. (1997). Influence of sampling on the reproducibility of ovarian follicle counts in mouse toxicity studies. *Repro Toxicol* **11**, 689–96.
- Davis, B. J., Dixon, D., and Herbert, R. A. (1999). Ovary, oviduct, uterus, cervix, and vagina. In: *Pathology of the Mouse* (R. R. Maronpot, ed.), pp. 409–443. Cache River Press, Vienna IL.

- Davis, B., and Heindel, J. (1999). Ovarian toxicants: multiple mechanisms of action. In *Reproductive and Developmental Toxicology* (K. Korach, ed.), pp. 373–395. Marcel Dekker, New York.
- Flaws, J. A., Doerr, J. K., Sipes, I. G., and Hoyer, P. B. (1994). Destruction of preantral follicles in adult rats by 4-vinyl-1-cyclohexene diepoxide. *Repro Toxicol* **8**, 509–14.
- Heindel, J. J. (1999). Oocyte quantitation and ovarian histology. In *An Evaluation and Interpretation of Reproductive Endpoints for Human Health Risk Assessment* (G. P. Daston and C. A. Kimmel, eds.), pp. 57–74. ILSI Press, Washington, DC.
- Hirshfield, A. N. (1994). Relationship between the supply of primordial follicles and the onset of follicular growth in rats. *Biol Reprod* **50**, 421–8.
- Hoyer, P. B., and Sipes, I. G. (1996). Assessment of follicle destruction in chemical-induced ovarian toxicity. *Ann Rev Pharmacol Toxicol* **36**, 307–31.
- International Conference on Harmonization (1994). Detection of toxicity to reproduction for medicinal products (S5A, finalized June, 1993). *Federal Register*, Vol. 59, No. 183, September 22, 1994, pp. 48746–52. (http://www.ich.org/UrlGrpServer.iser?@_ID=276&@_TEMPLATE=254).
- International Conference on Harmonization (1996). Maintenance of the ICH guideline on toxicity to male fertility: An addendum to the guideline on detection of toxicity to reproduction for medicinal products (S5B, amended Nov. 2000). *Federal Register*, Vol. 61, No. 67, April 5, 1996, pp. 15360. (http://www.ich.org/UrlGrpServer.iser?@_ID=276&@_TEMPLATE=254).
- Krupar, T. (1969). Oocyte destruction and ovarian tumorigenesis after direct application of a chemical carcinogen (9:0-dimethyl-1:2-benzanthrene) to the mouse ovary. *Inter J Cancer* **4**, 61–75.
- Mandl, A. M., and Zuckerman, S. (1952). Cyclical changes in the number of medium and large follicles in the adult rat ovary. *J Endocrinol* **8**, 341–6.
- Manson, J. M., and Mattson, B. A. (1992). Susceptibility of the ovary to toxic substances. In *Pathobiology of the Aging Rat* (U. Mohr, D. L. Dungworth, and C. C. Capen, eds.), Vol. 1, pp. 3365–76. ILSI Press, Washington, DC.
- Mattison, D. R. (1983). *Reproductive Toxicology*. Alan R. Liss, Inc., New York.
- Mattison, D. R., Shiromizu, K., and Nightingale, M. S. (1983). Oocyte destruction by polycyclic aromatic hydrocarbons. *Amer J Indus Med* **4**, 191–202.
- Morrissey, R. E., Schwetz, B. A., Hackett, P. L., Sikov, M. R., Hardin, B. D., McClanahan, B. J., Decker, J. R., and Mast, T. J. (1990). Overview of reproductive and developmental toxicity studies of 1,3-butadiene in rodents. *Environ Health Perspect* **86**, 79–84.
- Murphy, E. D., and Beamer, W. G. (1973). Plasma gonadotropin levels during early stages of ovarian tumorigenesis in mice of the W x -W u genotype. *Cancer Res* **33**, 721–3.
- Muskhelishvili, L., Freeman, L. D., Latendresse, J. R., and Bucci, T. J. (2002). An immunohistochemical label to facilitate counting of ovarian follicles. *Toxicol Pathol* **30**, 400–2.
- Organization for Economic Cooperation and Development (OECD) (1995). Reproduction/developmental toxicity screening test. In *OECD Guideline for Testing of Chemicals No. 421* (Adopted July 27th, 1995), pp. 1–10. OECD, Paris.
- Organization for Economic Cooperation and Development (2001). Proposal for updating Guideline 416: two generation reproduction toxicity study. In *OECD Guideline for Testing of Chemicals*, pp. 1–13. OECD, Paris.
- Peluso, J. J. (1992). Morphologic and physiologic features of the ovary. In *Pathobiology of the Aging Rat* (U. Mohr, D. L. Dungworth, and C. C. Capen, eds.), Vol. 1, pp. 337–49. ILSI Press, Washington, DC.
- Peluso, J. J., and Gordon, L. R. (1992). Nonneoplastic and neoplastic changes in the ovary. In *Pathobiology of the Aging Rat* (U. Mohr, D. L. Dungworth, and C. C. Capen, eds.), Vol. 1, pp. 351–64. ILSI Press, Washington, DC.
- Perez, G. I., Knudson, C. M., Leykin, L., Korsmeyer, S. J., and Tilly, J. L. (1997). Apoptosis-associated signaling pathways are required for chemotherapy-mediated female germ cell destruction. *Nat Med* **3**, 1228–32.
- Smith, B. J., Plowchalk, D. R., Sipes, I. G., and Mattison, D. R. (1991). Comparison of random and serial sections in assessment of ovarian toxicity. *Repro Toxicol* **5**, 379–83.
- Takizawa, K., Yagi, H., Jerina, D. M., and Mattison, D. R. (1984). Murine strain differences in ovotoxicity following intraovarian injection with benzo(a)pyrene, (+)-(7R,8S)-oxide, (–)-(7R,8R)-dihydrodiol, or (+)-(7R,8S)-diol-(9S,10R)-epoxide-2. *Cancer Res* **44**, 2571–6.
- Toaff, M. E., Abramovici, A., Sporn, J., and Liban, E. (1979). Selective oocyte degeneration and impaired fertility in rats treated with the aliphatic monoterpene, citral. *J Reprod Fertil* **55**, 347–52.
- U.S. Environmental Protection Agency (1998). Health Effects Test Guidelines, OPPTS 870.3800, Reproduction and Fertility Effects. EPA 712-C-98-239. U.S. Environmental Protection Agency, Office of Prevention, Pesticides and Toxic Substances, Washington, DC.
- U.S. Environmental Protection Agency (2000). Health Effects Test Guideline. OPPTS 870.3550. Reproduction/Developmental Toxicity Screening Test. EPA 712-C-00-367. U.S. Environmental Protection Agency, Office of Prevention, Pesticides and Toxic Substances, Washington, DC.
- U.S. Food and Drug Administration (2000). Redbook 2000. Toxicological Principles for the Safety Assessment of Food Ingredients. IV.C.9.a. Guidelines for Reproductive Studies. U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition, Washington, DC.
- Weitzman, G. A., Miller, M. M., London, S. N., and Mattison, D. R. (1992). Morphometric assessment of the murine ovarian toxicity of 7,12-dimethylbenz(A)anthracene. *Repro Toxicol* **6**, 137–141.
- Yuan, Y.-R., Foley, G. L. (2002). Female reproductive system. In *Handbook of Toxicologic Pathology* (W. M. Haschek, C. G. Rousseaux, and M. A. Wallig, eds.), Vol. 2, pp. 847–94. Academic Press, San Diego.