Review Article

The Rodent Estrous Cycle: Characterization of Vaginal Cytology and Its Utility in Toxicological Studies

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While an evaluation of the estrous cycle in laboratory rodents can be a useful measure of the integrity of the hypothalamic-pituitary-ovarian reproductive axis, it can also serve as a way of insuring that animals exhibiting abnormal cycling patterns are disincluded from a study prior to exposure to a test compound. Assessment of vaginal cytology in regularly cycling animals also provides a means to establish a comparable endocrine milieu for animals at necropsy. The procedure for obtaining a vaginal smear is relatively non-invasive and is one to which animals can become readily accustomed. It requires few supplies, and with some experience the assessments can be easily performed in fresh, unstained smears, or in fixed, stained ones. When incorporated as an adjunct to other endpoint measures, a determination of a female’s cycling status can contribute important information about the nature of a toxicant insult to the reproductive system. In doing so, it can help to integrate the data into a more comprehensive mechanistic portrait of the effect, and in terms of risk assessment, may provide some indication of a toxicant’s impact on human reproductive physiology. Birth Defects Res (Part B) 80:84–97, 2007. Published 2007 Wiley-Liss, Inc.

Key words: rodent estrous cycle; vaginal epithelial cell structure; toxicological studies

INTRODUCTION

The assessment of changes in vaginal epithelial cell structure has long been employed as a relatively non-invasive method to document reproductive cycles in laboratory rats and mice and to provide an index of the functional status of the hypothalamic-pituitary-ovarian axis. A few years after the early 20th century discovery by Stockard and Papanicolaou of rhythmic changes in the appearance of epithelial cells within the Guinea pig vaginal lumen (Stockard and Papanicolaou, 1917), Long and Evans published their landmark work characterizing the estrous cycle of the rat (Long and Evans, 1922). In the 1920s, the approach was also used by Allen and Doisy (1923) in ovariectomized mice for an early uterotrophic assay to assess the vaginal response following the administration of estrogenic hormones. Since then, the evaluation of such changes in epithelial cell structure in spontaneously ovulating laboratory animals has been used both as a principal measure in laboratory determinations of reproductive cyclicity and as an ancillary component of studies in Reproductive Toxicology. Consequently, as part of a revisitation of this approach, this report will focus on the various technical issues associated with performing such cytological assessments, the endocrine patterns that underlie the changes in vaginal cell morphology, and the contribution of these measures in determining the functional integrity of the female reproductive system. The concentration will be primarily on the rat, given its predominance as the species of choice in laboratory toxicology studies. However, similar changes in vaginal cytology do occur for cycling mammalian laboratory test species, although alternative methods, such as the appearance of a waxy, sulfur-containing vaginal discharge in the hamster on the day of estrus (Orsini, 1961; Singer et al., 1983) or measurements of vaginal electrical resistance (e.g., rats, Guinea pigs) (Bartos, 1977; Lilley et al., 1997; Ramos et al., 2001), have often been used to track cycles. Rats will typically begin cycling immediately after the vaginal orifice opens, which tends to occur between postnatal days 32 and 36. While there are some strain differences in the day of vaginal patency, rats may initially show some irregular cycles (Goldman et al.,

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Received 19 December 2006; Accepted 20 December 2006

The Research described in this article has been reviewed by the National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

Published online in Wiley InterScience (www.interscience.wiley.com) DOI: 10.1002/bdrb.20106

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costly. Alternatively, microscope slides can be substituted and archived, the use of such slides would be fairly 
Hollywood, CA). If the smears are to be fixed, stained, 
fixation or storage, we have found it preferable to use 12 
sufficient to evaluate a vaginal smear. 

Two-thirds and tapered. If a single dropper is used for more than 
one animal, it should be thoroughly rinsed between 
lavages to remove any residual cells from the dropper 
wall. When inserting the tip of the dropper into 
the vaginal orifice, it is important that the penetration 
be relatively shallow, approximately 1 cm. If it is too 
deep, this may provide excessive cervical stimulation, 
inducing a pseudopregnancy, which appears as a 
persistent diestrus. However, with care, the probability 
of this happening inadvertently is somewhat unlikely, 
since the cervix is positioned at a distance from the 
vaginal orifice. 

Normal saline. The dropper only need contain a 
small volume (0.2–0.25 ml) for flushing. Distilled water 
can also be used without markedly distorting the cells 
enough to impair identification. Only a drop or two 
needs to be placed on a slide. Smears can either be 
evaluated immediately, fresh and unstained, or fixed, 
dried, and stained for subsequent examination and 
storage (see below). 

Slides. If smears are to be evaluated fresh, without 
fixation or storage, we have found it preferable to use 12 
position raised ring slides (Fig. 1A) (Technical Glass, N. 
Hollywood, CA). If the smears are to be fixed, stained, 
and archived, the use of such slides would be fairly 
costly. Alternatively, microscope slides can be substituted 
and partitioned with a wax pencil (Fig. 1B). 

Microscope. A standard laboratory light micro-
scope with a 100 x total magnification is perfectly 
sufficient to evaluate a vaginal smear. 

CHARACTERIZATION OF THE VAGINAL SMEAR 

Identification of Vaginal Cytology 

Although a characterization of the vaginal smear has 
employed various terms over the years (e.g., Long and 

Evans, 1922; Hartman, 1944; Everett, 1989), the progression 
through a cycle essentially involves the regular recurrence of a few distinctive cell types, often 
in combination. The appearance of these cells typically 
correlates with the status of the vaginal mucosa, uterus, 
and ovaries and is linked to identifiable alterations in 
circulating concentrations of the sex steroids and 
gonadotropins. In a standard 4-day cycle, proestrus is 
identified by the presence of clusters of round, nucleated 
epithelial cells, which often have a granular appearance 
under the microscope (Fig. 2A,B). Occasionally, cellular 
strands may instead be present in the smear (Fig. 2C). 
Proestrus lasts for 1 day and is followed by vaginal 
estrus, routinely identifiable by the presence of large 
numbers of needle-like cornified (or keratinized) cells 
(Fig. 2D), or more rounded cells with jagged edges (Fig. 
2E). The predominance of cornified cells will last one day 
in a 4-day cycle, or can be present for 2 consecutive days 
in a 5-day cycle. Non-cornified epithelial cells (initially 
termed “pavement cells”) may alternatively be present 
during the period preceding leukocytic infiltration. 
Metestrus is a term that has been used to describe a 
transitional period during the early part of the first day 
of diestrus (diesirus 1), and its smear is characterized by 
a combination of leukocytes and cornified and rounded 
epithelial cells (Fig. 2F). These round epithelial cells 
commonly persist during days 1 and 2 of diestrus, when 
they co-occur with leukocytes in the smear (Fig. 2G). 
The concentration of leukocytes can vary, and the smear can 
ofen be almost exclusively leukocytic (Fig. 2H). The 
second day of diestrus (diesirus 2) may also show a few 
small clumps of nucleated epithelial cells (or some 
cellular strands) that will herald proestrus a day later. 
Some rats with a regular 5-day cycle may exhibit 3 
consecutive days of diestrus instead of the 2 days of 
cornification mentioned above. 

If samples are taken early in the day, one will often see 
a smear that will reflect a transition between the current
Fig. 2. Representative wet, unstained vaginal smears obtained on different days of the rat (Long-Evans hooded strain) estrous cycle. A,B: Proestrus; cells tend to appear in clumps and have a granular appearance. C: Proestrus; cells can alternatively be present as strands. D: Estrus; classic keratinized, needle-like cells. E: Estrus; cells can alternatively appear rounded, with jagged irregular edges. F: Metestrus; a combination of round "pavement cells," some needle-like cells, and a few smaller leukocytes can be present during a transitional period during the early portion of the first day of diestrus. G: Diestrus; leukocytes can appear in combination with various larger rounded cells. H: Diestrus; classic leukocytic smear with a few larger round epithelial cells.
day and previous one. The same is true for a sample taken later in the day, when cells characteristic of the forthcoming day are beginning to appear. For this reason, it is advisable to perform the vaginal lavages at approximately the same time each day.

Vaginal Keratinization: Relationship to Endocrine Alterations and Behavioral Receptivity

The keratinization of vaginal epithelial cells that typically characterizes the day of estrus is, in the cycling rat, a response to the rising level of estradiol that begins on the second day of diestrus and peaks around midday on proestrus (Fig. 3). These increasing concentrations act to up-regulate the brain hypothalamic mechanisms that amplify the pulsatile release of gonadotropin-releasing hormone into portal vessels that descend to the anterior pituitary. This rise in estradiol also sensitizes the pituitary, and it is this combination of effects on the hypothalamus and pituitary that triggers the ovulatory surge of luteinizing hormone. The surge, in turn, sets in motion the final stages of oocytic and ovarian follicular maturation that precede the ovulatory rupture of the follicle, an event that in the rat follows the surge by 10–12 hours.

One cautionary note should be mentioned here. The presence of a regular vaginal cycle does not necessarily indicate that ovulation has occurred. The luteinization of a follicle (i.e., formation of a corpus luteum) that follows release of the oocyte can also take place in unruptured follicles. For example, such luteinized unruptured follicles (LUFS) have been seen following treatment with anti-inflammatory agents in both rodents and humans (Killick and Elstein, 1987; Walker et al., 1988; Priddy et al., 1990).

The action of estradiol on epithelial vaginal cell keratinization is mediated by a spike in the local uptake of calcium (Gupta et al., 1990) and an induction of the calcium-dependent enzyme transglutaminase (Vijayalakshmi and Gupta, 1994), which accelerates the formation of covalent cell cross-links. This process also appears to involve the prompt initiation of an estradiol-induced increase in inositol phosphate turnover (Singh and Gupta, 1997) that acts to stimulate the calcium influx.

By definition, the term "estrus" refers to the period of heat or sexual receptivity. While it had been initially reported that receptivity paralleled the beginning of complete cornification of the vaginal smear (Long and Evans, 1922; Ball, 1937), the female was later found to be sexually receptive at the time when small numbers of cornified cells begin to appear (Young et al., 1941; Rodgers, 1970; Hardy, 1972), which occurs during the latter portion of the day of vaginal proestrus. Receptivity during the daylight hours of vaginal estrus is essentially absent. However, in females displaying a persistent vaginal cornification, sexual receptivity can be present (see Application of Vaginal Cytological Assessments in Toxicological Studies).

4- Versus 5-Day Cycles. While the surge in LH (along with a rise in follicle-stimulating hormone, FSH) occurs during late afternoon of the day of proestrus, there can be a disjunction between the presence of nucleated, granular epithelial cells (typically identifying

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**Fig. 3.** Schematic pattern of the 4-day estrous cycle in the rat depicting serum estradiol and progesterone concentrations as they relate in time to the surge of luteinizing hormone (LH). Ovulation will typically occur during the early morning hours of estrus, approximately 10–12 hours after the rise in LH. Shaded blocks at the base of the figure indicate the dark portion of a 14:10 hr light:dark photoperiod.
the day of proestrus) and the appearance of the surge. In rats that exhibit 5-day estrous cycles, characterized by two consecutive days of vaginal cornification, the gonadotropin surges actually appear on the first cornification day and not on the apparent day of vaginal proestrus (e.g., Kaneko et al., 1986; Hashimoto et al., 1987; Everett, 1989). Ovulation then takes place during the early morning hours of the second day. Table 1 shows oocyte yields for both types of estrous cycles when rat oviducts were flushed on the first or second day of cornification, indicating a 5-day cycle at that time. All regular 5-day females had oocytes present on the second day, while the oviducts of the two rats flushed mid-day on the first day of a cornified smear were empty. These shifts between 4- and 5-day cycles can be particularly vexing when, in such assessments of oocyte release, animals are euthanized on the first day a fully cornified smear is observed. Oviducts flushed from a female with a ballooned uterus (indicative of an endocrine proestrus) smear is observed. Oviducts flushed from a female with ethynyl estradiol (0.01 and 0.1 mg/kg, 25 days), Laws et al. (2000) reported the initial appearance of a pseudopregnancy followed by a continuously cornified smear. The pseudopregnancy was attributable to an estrogen-stimulated increase in prolactin secretion, which in turn rescued and maintained functional corpora lutea. It essentially refers to an endocrine state characteristic of a pregnancy but without implantation and embryonic development. Cyclicity is suppressed, with a persistence of leukocytes in the vaginal smear. In the rat, both diurnal and nocturnal surges of prolactin maintain corpora luteal function (a luteotrophic effect confined primarily to rodents), whereby elevated levels of progesterone are secreted for approximately 12–14 days before luteolysis ensues (Fig. 4). With a prolongation of treatment, the prolactin surges will cease, progesterone secretion declines, and the smear will become cornified, typically due to atrophic changes in the ovaries resulting from diminished gonadotropin secretion, a likely consequence of the effect of the estrogen or estrogenic compound on the brain and pituitary. One of the frequently employed approaches to summarize cyclicity has been to determine the percentage of days in estrus or diestrus within a treatment group over a given period of time. If the effect of toxicant exposure on the cycle is consistent within a group (e.g., induction of persistent estrus), then this can provide useful summary data. However, should exposure result in some animals showing a prolonged diestrus, while others exhibit persistent estrus, then such group summaries may not reflect these changes and the differential effects in aggregate could result in an overall impression that cyclicity has not been affected.

Cycles may also be dichotomized as regular or irregular, with regular being defined as a 4- or 5-day cycle with 1 or 2 days of estrus or one with a single day of estrus and 2 or 3 consecutive days of diestrus. Such a dichotomy would then lump animals having irregular 6 or 7-day cycles with those exhibiting a persistent estrus or diestrus. For such classifications, we have found it more helpful to identify cycles as regular (4 or 5 days with either 1–2 days of estrus or 2–3 diestrus days), extended (3–4 days of estrus or 4–5 days of diestrus), or abnormal (>4 consecutive days of estrus or >6 days of diestrus).

A disruption of cycling caused by xenobiotic treatment can induce an acyclicity characterized by a persistent estrus, a persistent diestrus, or cause an irregular pattern with cycles of extended duration. Exposure to estrogenic compounds has commonly been reported to result in a persistent vaginal cornification (e.g., Jones and Edgren, 1973; Brawer et al., 1978; Kumagai and Shimizu, 1982; Yoshida et al., 2000). Lower dosages have been found to induce a persistent diestrus or a pseudopregnancy (e.g., Bogdanove, 1966; Gilmore and McDonald, 1969; de Greef and Zeilmaker, 1979). Using daily oral exposures to ethynyl estradiol (0.01 and 0.1 mg/kg, 25 days), Laws et al. (2000) reported the initial appearance of a pseudopregnancy followed by a continuously cornified smear. The pseudopregnancy was attributable to an estrogen-stimulated increase in prolactin secretion, which in turn rescued and maintained functional corpora lutea. It essentially refers to an endocrine state characteristic of a pregnancy but without implantation and embryonic development. Cyclicity is suppressed, with a persistence of leukocytes in the vaginal smear. In the rat, both diurnal and nocturnal surges of prolactin maintain corpora luteal function (a luteotrophic effect confined primarily to rodents), whereby elevated levels of progesterone are secreted for approximately 12–14 days before luteolysis ensues (Fig. 4). With a prolongation of treatment, the prolactin surges will cease, progesterone secretion declines, and the smear will become cornified, typically due to atrophic changes in the ovaries resulting from diminished gonadotropin secretion, a likely consequence of the effect of the estrogen or estrogenic compound on the brain and pituitary. One of the frequently employed approaches to summarize cyclicity has been to determine the percentage of days in estrus or diestrus within a treatment group over a given period of time. If the effect of toxicant exposure on the cycle is consistent within a group (e.g., induction of persistent estrus), then this can provide useful summary data. However, should exposure result in some animals showing a prolonged diestrus, while others exhibit persistent estrus, then such group summaries may not reflect these changes and the differential effects in aggregate could result in an overall impression that cyclicity has not been affected.

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How Long Should Evaluations Be Performed?

The aberrant nature of young adult females exhibiting a persistent vaginal estrus is quite obvious. However, the significance of a small number of extended 6- or even 7-day cycles is a question open to debate. In an individual animal, is it biologically meaningful if one cycle of 6 or 7 days is observed over the course of 3 weeks? In and of itself, it may not be. The question needs to be considered within the context of the study, and the results referenced against those of controls and other exposure groups, since untreated animals displaying normal cycles may on occasion exhibit extended ones. If females in a treated group consistently show that 1 or 2 of 4 cycles are extended in duration as compared to none for controls, then this may well be meaningful. It then becomes important to characterize cyclicity over a sufficient number of days in order to allow one to identify any such alterations, if present. The U.S. Environmental Protection Agency has recommended including an evaluation of estrous cyclicity in its Reproductive and Fertility Effects Test Guidelines, OPPTS 870.3800 (U.S. EPA, 1998) for a multigenerational protocol. In doing so, it is intended that cytological information collected over the course of 21 days (or 4–5 cycles) prior to mating in both F0 and F1 females be used to complement other more traditional measures of toxicological impact.

Other Technical Issues

Blind versus non-blind assessments of vaginal cytology. In toxicology studies, one recurrent question in the evaluation of vaginal cytology has been whether smears should be read blind. There are actually two issues here: (1) a knowledge of treatment group assignments, and (2) whether daily smears should be recorded for each female without knowing the identity of previously recorded smears. A characterization of the vaginal smear should always be performed without an awareness of which treatments individual animals are receiving. However, for an accurate assessment of cycling status, it is important to see the pattern of changes within an individual animal, particularly since it is common to see smears that reflect a transition between days, for which multiple cell types are present. Without such information about the preceding days’ smears, it can be difficult to make the appropriate determination, thereby increasing the likelihood for error. For example, the second day of diestrus in a 4-day rat may have some cell clumps, each with a small number of nucleated epithelial cells. Knowing the previous days’ results will help one to identify the smear as diestrous rather than proestrus, particularly when cells on the succeeding day show clear evidence of proestrus. This is also true for an estrous smear, which may show a predominance of large rounded cells, instead of the typical cornified
Ones. Knowing that the previous day’s smear was proestrus would help identify it as estrus, rather than diestrus. Again the goal is to minimize errors and provide an accurate evaluation of the individual cycles, while maintaining ignorance of the treatment assignments.

**Fresh versus fixed and stained smears.** In many studies, vaginal smears are characterized unstained and viewed wet immediately after being obtained. Archival storage may not be required, as in some studies for which smears are taken only to establish general cycling status prior to experimental treatment. Cells can then be evaluated, data recorded and the smear slides rinsed clean.

For those studies incorporating an evaluation of estrous cyclicity as an endpoint measure, it may be necessary to preserve fixed and stained smears. A variety of procedures have been used over the years. Cells have been fixed either after the smear has been dried (e.g., Everett, 1989; Rhodes et al., 2002) or while still wet (e.g., Shorr, 1941; Svhila, 1944; Mandl, 1951), using various ethanol-based formulations. For general purposes of evaluation, the type of stain employed can vary considerably, although it is important that heavy staining not be used and excess be washed from the slides. Stains that have been employed include methylene blue, toluidine blue O, and hematoxylin. However, which of these is used would be of minor importance, as long as the color they impart allows accurate identification of the cell type. Some examples of smears stained with toluidine blue O are provide in Figure 5. The cells were fixed with 95% ethanol and dried. Smears were subsequently de-salted with a distilled water rinse and stained for 4 min in an aqueous solution of 0.1% toluidine blue O made slightly basic with dilute sodium hydroxide. The slides were then rinsed again and air dried.

**Recording and analyzing cycling data.** Since the cells in a good percentage of smears are not uniform in appearance and often reflect a transition between different phases of the cycle, it is important to record what one sees. The relative abundance of each cell type can be specified using a rough estimate of scans across the smear (Fig. 6A), or a non-quantitative characterization made based upon a subjective assessment of the smear (Fig. 6B). If a diestrous smear contains a few small groups of nucleated epithelial cells, it should be indicated. The day can then be recorded as \( \text{D(p)} \), indicating that while the day is diestrus, the presence of the nucleated, granular cells (entered as the \( \text{p} \) in parentheses) is a sign that tomorrow will likely be the day of proestrus. The designation \( \text{P(e)} \) refers to the beginning of a similar transition, in which cornified epithelial cells begin to appear during the latter portion of proestrus. Regardless of the way in which the cells are documented, the smears should be taken during approximately the same time each day, and in recording the information, it is important that the smear be scanned throughout for an accurate characterization.

When plotting the data, it is helpful to depict cyclicity in such a way that a repetitive pattern and deviations from this pattern are more readily recognizable. If the days are plotted as single letters, it is helpful to place them in corresponding colored or shaded blocks (Fig. 7A). The cycles can also be represented as a sequence of connected dots (Fig. 7B) or columns of different heights
(Fig. 7C), with diestrus the lowest, proestrus in the middle, and estrus at full height. Using the latter approach, an example is presented of a 14-day exposure to a hypothetical xenobiotic preceded by a 2-week pre-treatment period and followed by a similar non-exposed post-treatment period (Fig. 8). Figure 8 includes illustrations of both persistent estrus and presumptive pseudopregnancies.

Alterations in estrous cycles have been analyzed in a number of different ways. One commonly employed approach has been to record for each animal in a treatment group the total number of days of vaginal estrus over a specified period of time. Group data are then subjected to analysis of variance, with comparisons made between pre- and post-treatment periods or to concurrent controls. However, in this approach, as mentioned previously, if a particular treatment shows a mixed effect within a group, both extended episodes of estrus and diestrus, the combined group effects of exposure may mask an effect, statistically canceling one another out. Average cycle length or the number of cycles over a prescribed number of weeks are two forms of the same categorization. A cycle can then be identified as the number of days between the beginning of full vaginal cornification until the beginning of the next one. Alternatively, one can define a cycle as the number of days from one proestrus to another. However, quantifying the number of cycles over a defined period of days could conceivably obscure some toxicant-induced effects. Figure 9 shows 2 examples of a 21-day cycling pattern. In each, there are four cycles with nine identified days of estrous vaginal cell cornification, or 43% of the total. While the top cycle, with the exception of a single 3-day period of estrus, shows a more normal 5-day pattern, the lower one exhibits an extended 6-day sequence of cornification that may well persist beyond the 21-day smear period, a pattern that in response to chemical exposure would clearly reflect an adverse effect.

A third option, reiterated from above, involves a categorization of cycles as regular (4- or 5-day cycles), extended (3–4 consecutive days of estrus, or 4–5 days of diestrus), or abnormal (>4 days of estrus or >5 days of diestrus). These categorical data can then be compared by chi-square analysis. At this time, it is also useful for descriptive purposes to classify separately the type of abnormality (persistent estrus or persistent diestrus) within treatment groups, since diverse classes of compounds may differentially affect the cycles, depending
upon the nature of their impact on the mechanisms of endocrine regulation.

APPLICATION OF VAGINAL CYTOLOGICAL ASSESSMENTS IN TOXICOLOGICAL STUDIES

In toxicological studies, data provided by a characterization of the estrous cycle, either as a principal endpoint or as an adjunct to other measures, is useful for a variety of different reasons.

In Evaluations of the Functional Integrity of the Hypothalamic-Pituitary-Ovarian Axis Following Toxicant Exposure

As previously discussed, alterations in the endocrine relationships among the hypothalamic, pituitary, and ovarian components of the reproductive axis can have marked effects on cyclicity. A toxicological insult to any one of these sites can disrupt the cycle and block ovulation. Moreover, the pattern of alterations in the cycle can frequently provide valuable information about the nature of a compound’s effect on the reproductive system.

In toxicology studies, measurements of sex steroid concentrations can be markedly affected by cycling status, and it becomes critical to incorporate cyclicity data when such endocrine assessments are made. For example, an alteration in the serum concentrations of estradiol and progesterone seen in blood taken at necropsy following xenobiotic exposure may not necessarily be a consequence of a direct effect on the steroid pathway. It may in effect, however, be secondary to the female becoming acyclic or the presence of a repetitive pseudopregnancy.

Compounds that have been reported to destroy or impair the growth of ovarian follicles can have marked effects on cyclicity. 4-vinylcyclohexene diepoxide, a chemical used in the production of epoxy resins, has been found to deplete the population of preantral follicles in both rats and mice (Smith et al., 1990; Kao et al., 1999). Compared to controls, cycling is disrupted over time in treated rats (Mayer et al., 2002) and mice (Lohff et al., 2005), although the effect did not appear to be attributable to a decline in proestrus levels of estradiol (Lohff et al., 2005), even though circulating concentrations of FSH did rise, something that is typical in rodents following gonadectomy (e.g., Frager et al., 1981). Similar disruptions of cyclicity and an effect on follicular growth have been reported following exposure to 1-bromopropane (Yamada et al., 2003), 2-bromopropane (Kamiyama et al., 1997), and 1,2-dichloropropane (Sekiguchi et al., 2002), which are used as cleaning solvents in electronic industries.

Acute exposures to compounds that interfere with processes within the hypothalamus that are involved in stimulating GnRH secretion can block the initiation of the

Fig. 7. Some examples of graphically expressed cycling data. A: Shaded blocks lettered with the individual days of the cycle. B: Connected dots with the lower level dots for each animal indicating diestrus, the middle level dots indicating proestrus, and the highest level dots indicating estrus. C: Columns of different heights with the 1/8 height, 1/2 height, and full height columns representing diestrus, proestrus, and estrus, respectively.
LH surge and alter cycling status. The neurotransmitter norepinephrine, which is secreted in the hypothalamic region, acts as a permissive factor in GnRH release. An inhibition of norepinephrine synthesis or a noradrenergic receptor blockade in the hours prior to the initiation of the rise in LH will block the surge. Pesticidal dithiocac-
rbamates act as metal chelators and bind the copper cofactor necessary for the activity of dopamine-β-hydroxylase, the enzyme generating norepinephrine from dopamine. Daily exposure to these compounds (Cooper et al., 1999; Murr et al., 2006), along with the application of other norepinephrine-depleting measures (Clifton and Sawyer, 1979; Simpkins et al., 1979), will cause disruptions in cyclicity, although the system, in spite of continued dosing, is able to adapt after a period of time and resume cycling and normal ovulation. Although such compensatory alterations to toxicant insult may not necessarily be more broadly applicable to xenobiotic exposures in general, these changes do speak to the use of extended exposure paradigms in assessments of toxicological effects on reproductive functions. Consequently, an assessment of estrous cyclicity over 2-week segments following the initiation of treatment, can indicate the emergence of an effect on the reproductive system that may either be short-lived or persistent.

Inclusion of an evaluation of vaginal cytology is particularly meaningful in studies of reproductive aging. In chronic exposure studies, an awareness of how ovarian cyclicity may be altered with age is important, since most rat strains show an age-associated disruption in cycling between 10 and 12 months of age, and various toxicants have been reported to induce a premature reproductive senescence. Dietary exposure to the chlorotriazine herbicides has been shown in postpubertal females to cause a premature pattern of persistent estrus (Eldridge et al., 1994; Stevens et al., 1994). Also, a brief perinatal exposure to estradiol (Gorski, 1977) or estrogenic compounds such as methoxychlor (Gray, 1991) was found to induce an early cessation of cyclicity. Rats administered a high prepuberal dose of estradiol showed a precocious vaginal opening that was promptly followed by a persistent vaginal cornification (Gorski, 1977). At lower dosages of estradiol, the onset of persistent cornification was delayed until a later age (a delayed anovulatory syndrome).

To Insure Pretreatment Comparability Among Treatment Groups

For any evaluation of the effects of xenobiotic exposure on reproductive function, it is important to insure that test animals are cycling regularly prior to the initiation of treatment. One can not automatically assume that newly acquired females are 4- or 5-day cyclers, and it is not infrequent to find irregularities in an unwanted percentage of arriving animals. If females are confirmed to be cycling regularly before any exposures are conducted, any immediate effects of treatment can be identified and comparisons made both with sham-dosed control animals and with the pre-exposure period.

Xenobiotic exposure during a particular day of the cycle (and time during that day) can have a marked impact on the endocrine regulation of reproductive function. The ovulatory surge of LH on proestrus has been found to be particularly vulnerable to compounds that target hypothalamic activity, when exposure to these compounds occurs during a sensitive window of time that encompasses those hypothalamic processes that serve to stimulate the surge. This window occupies a period of time approximately 3–5 hours before the late afternoon rise in LH. Exposures taking place outside of this window may then be much less effective (Goldman et al., 1991, 1994; Stoker et al., 1993). Consequently, understanding the relationship between the time of exposure and the day of the cycle is an important component, in the adult female, of any assessment of the reproductive impact of compound administration.

In Mating Studies

In multigenerational protocols, cohabitation of males and females typically extends over 1 to 2 weeks. Upon ejaculation, secretion from the male rat accessory glands forms a copulatory plug in the female’s vagina, and its presence is taken as evidence that mating has occurred. However, a significant percentage of nocturnally-formed plugs will dislodge before the females are examined (Szabo et al., 1969), which normally takes place during the following morning. If the plugs cannot be found in a solid bottom cage or a feces pan, vaginal smears are often taken. Although the presence of sperm, not cytological assessments, is the primary consideration, an increased interval between the start of cohabitation and such evidence of mating suggests a potential alteration in estrous cyclicity and/or behavioral receptivity.

Animals in persistent estrus can also be sexually receptive (Cooper and Linnoila, 1977) and exhibit a mating-induced LH surge (Brown-Grant et al., 1973; Matt et al., 1987; Day et al., 1988). However, the percentage of such females that become pregnant is considerably reduced (Cooper and Goldman, 1999). Neonatal androgenization will cause an emergent persistent estrus and has been used in the past as a model to investigate fertility in such anovulatory animals mated following an induced ovulation. The data have been somewhat equivocal (e.g., Witschi and Pfeiffer, 1935; Ericsson and Baker, 1966; Dorner and Fatschel, 1970; Sawada, 1987), but an observed decline in fertility in these persistent estrus females has been reported to be attributable to the size of the initial dose of androgen employed (Kramen and Johnson, 1971), or the lack of sufficient priming (Sawada, 1987). Nevertheless, in a toxicology study, it would be unclear if any effects on litter size in those pregnant females previously exhibiting a persistent estrus were due to a direct effect of toxicant exposure, to the anovulatory status prior to a mating-induced ovulation, or a combination of both. Given this possibility, it becomes important that the cycling status be verified prior to the period of cohabitation.

To Insure That Animals Are Euthanized at the Same Time in Their Cycles, or to Place Into Context Endocrine Measures From Those Euthanized on Different Days of the Cycle

For studies involving endocrine and uterine assessments, the relationship between the two parameters can be critical. On the day of endocrine proestrus, the weights of both fluid-filled and fluid-evacuated uteri will be considerably elevated. Thus, depending on the endpoints under study, the data can show a considerable degree of variability if they are obtained at different times of the cycle, or from a treatment group comprised of both cycling and non-cycling females. Such an increase in intra-group variability could lead to a type-2 statistical error, for which there is a failure to find a treatment effect when it actually exists.
The Utility of Single-Smear Data

Characterization of an animal’s cycling status derived from a single smear is generally of limited value. While the information can contribute to an identification of the status of the reproductive system at necropsy, other measures (e.g., organ weights, ovarian and uterine histology, endocrine assessments) are needed for a more conclusive determination of ovarian status. In studies of mating behavior, or those involving post-mating gestational exposures, a single identified proestrus smear can be used in the process of selection to improve the likelihood that mating will occur later that day or on the morning of vaginal estrus, thus improving the pregnancy rate. As discussed above, the presence of a copulatory plug or sperm in a vaginal lavage on the morning of estrus would then confirm that mating had taken place.

In consideration of the influence of toxicant exposures on cyclicity, evaluation of a single smear may only be of some use when the results are considered in group cross section. On any one day, vaginal smears taken from control females can be expected to show a cytological variety that reflects a typical asynchronicity of cycles within the group. On the other hand, toxicant-exposed animals may show a greater uniformity of cells across the group that suggests a common effect of treatment on the endocrine status. Nevertheless, the interpretation of such data should only be done within the context of other endpoint measures.

RELATIONSHIP TO HUMAN HEALTH

RISK ASSESSMENT

While evaluations of vaginal cytology in the laboratory rodent can provide a valuable reflection of the integrity of the hypothalamic-pituitary-ovarian axis, other indices are more useful in humans to determine the functional status of the reproductive system (e.g., menses, basal body temperature, alterations in vaginal pH, cervical mucus viscosity, and blood hormone levels). Nevertheless, since many of the mechanisms involved in the regulation of the reproductive axis are similar across species (particularly those mediated through the estrogen receptor), assessments of rodent estrous cyclicity can offer insight into potential adverse effects in humans.

Common classes of chemicals have been shown to cause cycle irregularities in rats, humans, and nonhuman primates. Occupational estrogen exposure of female employees in companies formulating oral contraceptives has been reported to cause cycling irregularities among other symptoms of hyperestrogenism (Harrington et al., 1978), while effects in rodent species are well-documented (e.g., Gilmore and McDonald, 1969; Jones and Edgren, 1973; Brawer et al., 1978; Hayashi and Moberg, 1990). Other examples include the polychlorinated biphenyls (PCBs) and dioxins, which are also associated with such irregularities in rats and humans (Brezner et al., 1984; Li et al., 1995; Meerts et al., 2004; Yang et al., 2005; Chao et al., 2007). This also appears to be true for exposure to mercury (e.g., Stadnicka, 1980; Sikorski et al., 1987; Yang et al., 2002; Davis et al., 2001) and various agricultural pesticides, including herbicides, fungicides, and fumigants (Heinrichs et al., 1971; Gotz et al., 2001; Windham et al., 2005; Farr et al., 2004).

It should be acknowledged that reports of these effects on cyclicity in women are frequently fraught with potential confounding factors, including general medical condition and medications taken, alcohol consumption, socioeconomic status, and age. Nevertheless, what is significant within the context of this discussion is that, although the assessment of the human ovarian cycle may have a variety of biomarkers distinct from those in rats, many of the underlying endocrine mechanisms associated with successful follicular development, ovulation, pregnancy, and parturition are homologous between the two (for a recent review, see Bretveld et al., 2006). For this reason, a toxicant-induced inhibition of ovarian cycles in female rats should certainly suggest that a compound may function as a reproductive toxicant in human females. However, exposure to environmental compounds may alternatively induce irregular or moderately extended ovarian cycles. These latter changes should be interpreted with caution and not judged adverse without a comprehensive consideration of additional relevant endpoints in a weight-of-evidence approach.

SUMMARY

While an evaluation of the estrous cycle in laboratory rodents can be a useful measure of the integrity of the hypothalamic-pituitary-ovarian reproductive axis, it can also serve as a way of insuring that animals exhibiting abnormal cycling patterns are disincluded from a study prior to exposure to a test compound. Assessment of vaginal cytology in regularly cycling animals also provides a means to establish a comparable endocrine milieu for animals at necropsy. The procedure for obtaining a vaginal smear is relatively non-invasive and is one to which animals can become readily accustomed. It requires few supplies, and with some experience the assessments can be easily performed in fresh, unstained smears, or in fixed, stained ones. When incorporated as an adjunct to other endpoint measures, a determination of a rodent’s cycling status can contribute important information about the nature of a toxicant insult to the reproductive system. In doing so, it can help to integrate the data into a more comprehensive mechanistic portrait of the effect, and in terms of risk assessment, may provide some indication of the capacity of a toxicant to influence human reproductive physiology.

ACKNOWLEDGMENTS

The authors are grateful for the continuing support of Yolanda Autry and Al Moore (Charles River Laboratories), and Faye Poythress, Svetlana Kilmon, Yesensia Castro, and Guillermo Roozco (First Priority Service) in providing daily vaginal lavages over the course of various studies. We also thank Janet Ferrell for her assistance and Dr. Suzanne Fenton for providing helpful comments on an earlier draft of the manuscript.

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