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Monitoring of the Estrous Cycle in the Laboratory Rodent by Vaginal Lavage

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Introduction

Ovarian cyclicity in a number laboratory species can be monitored easily and noninvasively by observing changes in the vaginal cytology. This process has been successfully employed by numerous researchers for more than 70 years, since the initial studies published by Stockard and Papanicolaou (1) in the guinea pig, Allen (2) in the mouse and Long and Evans (3) in the rat. Information obtained from the daily vaginal smear not only provides a simple means to determine the normal pattern of ovarian activity present in the different species, but also allows one to determine the extent to which normal function may be disrupted by toxicant exposure.

The readily identifiable and predictable changes in the cytological make-up of the vaginal smear occur as the consequence of the patterned and dramatic fluctuations of blood estradiol concentrations that are initiated at puberty and continue (unless interrupted by pregnancy, environmental or experimental insult) until reproductive senescence. In fact, reproductive senescence in the rat is characterized by the vaginal smear pattern that predominates in later life (e.g., constant estrus or repetitively pseudopregnant).

This relationship between ovarian estrogen and vaginal cytology, as it exists in the 4-day cycling female rat, is outlined in Figures 1 and 2. During the periods of diestrus (as well as pregnancy and pseudopregnancy) blood estrogen concentrations are low and the vaginal smear contains a mixture of

cell types with a predominance of leukocytes and a few scattered cornified epithelial cells (figure 2a). The increase in serum estradiol concentrations (originating from the maturing follicles) has a proliferative action on the vaginal epithelium. On proestrus, this action of estrogen on the epithelium produces a smear with a predominance of round, polynucleated cells that may be dispersed or clumped (figure 2b1 and 2b2). As serum estradiol levels fall (coincident with ovulation and formation of the corporal lutea), the proliferation of the vaginal epithelium declines (figure 2c). At this time, cornified epithelial cells are predominant in the smear. Not depicted in Figure 1 is the transitional stage between the period of estrus to diestrus in which there is a large number of both cornified cells and leukocytes. This period, identified as metestrus by many investigators, is brief and different forms of cornified cells may be recognized, ranging from the typical jagged shape to a highly rounded form such as that depicted in figure 2d. However, the presence of leukocytes usually indicates the transition out of estrus. Identification of these different cell types and the pattern of change occurring during the estrous cycle are best determined by following individual females for a number of consecutive days.

The relationship between ovarian cyclicity and vaginal cytology is similar in the house mouse to that in the rat (figure 3). However, some differences should be noted. Vaginal opening, which occurs about day 23 in the mouse, does not represent puberty because several days or even weeks can

pass before a fully cornified smear appears, signalling first ovulation. The cycle, once underway, averages about 5 days, but is far from regular. Some of this irregularity may be ascribed to social conditions. Female mice housed in the same room with males tend to cycle more regularly, while females housed in all female groups can remain anestrous for weeks (4, 5). Nevertheless, under a controlled environment, regular daily lavages of the vagina will harvest cells indicative of the stages of the ovarian cycle and will signal ovulation (6, 7).

It has been suggested that the ovarian cycle of the female can be monitored by changes in the external appearance of the vagina (8), e.g., the vaginal orifice has a gaping appearance at estrus. While these changes can be generally correlated with the various stages of the estrous cycle, by themselves they have not proven to be a useful measure in the mouse (Vandenbergh, unpublished observations).

Procedures

Consistent ovarian cycles can be detected in female rodents only if they are housed under regular lighting conditions. Standardized photoperiods have defined by either a 14h:10h or 12h:12h light:dark schedule. Major deviations from these photoperiods are known to lead to alterations in the regularity of the female's cycle. For example, constant light will lead to a disruption of ovulation and the appearance of a persistent or constant vaginal estrus. Under standard lighting conditions, estrous cycles will be observed and these cycles

will continue in a rhythmical fashion throughout adulthood (until approximately one year of age) unless the female is mated or treated experimentally. In addition to maintaining a proper lighting schedule, the smears should always be collected at the same time of day.

Studies with Peromyscus mice (9) and hamsters (10, 11) suggest that even brief flashes of light during the dark phase of the day can reset internal pacemakers and can have an effect on reproductive rhythmicity. To be cautious, laboratory colonies of rodents should not be transiently exposed to brief periods of light (e.g., door opening or lights on briefly) during the dark portion of the photoperiod. The animals respond to differences in light intensity, not the absolute presence or absence of light. Consequently, if nocturnal inspection is required by the experimental protocol, very dim or red light can be left on at night.

Obtaining a smear is a simple process. Using a clean microscope slide (e.g., serological ring slide, Scientific Products, Catalog number M6229-1) and an eye dropper containing approximately 0.25 ml of distilled water or physiological saline, the rat's vaginal cavity is thoroughly lavaged and the fluid is drawn back into the eye dropper (figure 4). If the initial flush is devoid of cells, the lavage is repeated. Caution should be exercised to ensure that only the tip of the eye dropper is inserted into the vaginal cavity, so that stimulation of the uterine cervix is avoided. The collected fluid is then expelled evenly onto the microscope slide in a thin layer. The smear may be

viewed immediately under low magnification (e.g., 100 x).

Because of the smaller size of the mouse, a Pasteur pipet (e.g., Fisher catalog no. 13-678-6A, 15 cm long) rather than an eye dropper should be used. To protect the vagina of the mouse from injury, the tip of each pipet should be briefly fire polished to smooth jagged edges. The pipet is fitted with a rubber bulb and a small quantity of saline is flushed 2-3 times in the vagina to harvest the contents.

Daily vaginal smears should be collected from each female for a period of at least two weeks, so that the cycling pattern can determined accurately. Most investigators do this by recording the smears as they are taken on a daily basis and keeping continuous records on each female. This approach also provides the novice with the opportunity to observe ongoing fluctuations in the various cell types as they occur. An alternative approach is to obtain the daily smears and read them at a later time. In this case, the smears are arranged in order on the microscope slide, allowed to dry, and periodically fixed and stained. One technique used by Everett (12) is to stain at the end of each week in 1% toluidine blue O (Fisher Scientific, Cat # T-161) after fixation in 95% ethanol and removal of salt by washing in deionized water. The dye solution must be neutral or slightly alkaline, or the cornified cells will remain colorless. Nuclei and mucous are stained metachromatically pink. The cytoplasm appears in various shades of blue ranging from very dark, in case of small epithelial cells, to pale blue in the squamous cells, whether on not the

latter are cornified. While staining is not essential to evaluate the smear, for training purposes application of a dilute vital stain such as methylene blue to the smear is helpful in initially characterizing the cell types. Once noted, they are easily recognizable and staining is no longer necessary.

COMMENTS

Evaluation of Cyclicity in Toxicological Studies

Monitoring the vaginal smear of the nonpregnant female provides a useful ancillary measure in toxicology studies. Properly used, this information can serve to reduce much of the variance associated with a variety of measures that fluctuate over the cycle. Furthermore, characterization of the vaginal smear pattern offers information about ovarian status prior to treatment and provides a way to make certain that only regularly cycling females are assigned to control and experimental groups (e.g., usually defined as the presence of consecutive cycles for 2-3 weeks prior to study). Regardless of the strain of rat or mouse, regular cycling may not necessarily occur in all young-adult animals. The incidence of aberrant vaginal smear patterns (indicative of altered ovarian activity) may be as high as 20-30% with certain commercial shipments, while at other times it may be as low as 5%. In either case, including non-cycling animals in an experiment, even when randomly assigned to treatment groups, could well increase the variance for many measures.

Knowledge of the vaginal smear prior to treatment also provides a point

of comparison immediately after the initiation of treatment and for any subsequent changes that may ensue. In chronic studies, an awareness of how ovarian cycling is altered with age is important, as most strains show an age-related disruption of cyclicity at approximately 12 months of age. For example, female Long Evans hooded rats assume a pattern of constant vaginal estrous at this age. Thus, treatment-induced alterations in this normal, age-associated alteration in cycling would be important.

Another reason for keeping a daily record of cycling status would be to synchronize the time of data collection. By killing the females at the same stage of the cycle, variability inherent in the ovarian cycle would be minimized and the probability of detecting an effect would be enhanced. For example, euthanasia at specific times on the day of vaginal proestrus would yield information about the preovulatory rise in circulating levels of estrogen, progesterone and luteinizing hormone and/or the status of the preovulatory follicle. An examination of the females during late estrus/early diestrus (metestrus) would allow one to assess the presence of fresh corpora lutea and whether or not ovulation has occurred (e.g., see Perreault and Mattson, this volume).

Daily vaginal smear data also provide the following types of information: (a) presence of a copulatory plug or sperm after mating. In the mouse, for example, a copulatory plug is found by daily inspection of the vagina in approximately 80% of breeding females. (b) determination of

pregnancy (or continuation of cycling after mating), (c) distinguishing pregnancy from pseudopregnancy, based on the number of days smear remains leukocytic (e.g., pseudopregnancy = approxximately 14 days, pregnancy = 21-22 days), and (d) indications of fetal death and resorption by the presence of blood in the smear after day 12 of gestation.

In studies evaluating the effect of a single treatment on female reproductive function, knowledge of the animal's cycling pattern is critical, as a toxicant may affect the reproductive process differently at any of the numerous stages of follicular development, ovulation, egg transport and implantation. For example, ovulation may be advanced by treatment with estrogen on diestrus I (13). On the other hand, treatment with various compounds on the day of vaginal proestrus, administered during the so-called critical period, have been shown to affect the characteristics of the LH surge, and subsequently ovulation (see chapter by Goldman and Cooper, this volume). Anesthetics (12), pharmacological compounds that alter brain noradrenergic or cholinergic neurotransmission (12, 14) and pesticides (15, 16) have all been shown to block the LH surge and delay ovulation (Goldman and Cooper, this volume). Single treatments with the same dose of these compounds at other times during the estrous cycle are without effect on the LH surge or ovulation. In the cycling female, the vaginal smear is used to determine when the female's oviduct can be examined for the presence of oocytes (i.e., on the morning of vaginal estrous). Oocytes may not be detected

in cycling animals in whom (a) the timing of the LH surge has been altered (17), (b) follicular rupture has been blocked without affecting luteinization (18), and (c) the rate of oocyte transport has been altered by treatment with compounds such as the weakly estrogenic pesticide, methoxychlor (19).

Compound-Induced Disruptions in Cyclicity

A compound that disrupts ovarian cycling (and thus the female's fertility potential) could induce a pattern of constant vaginal estrus, repetitive pseudopregnancies or anestrus conditions. Constant vaginal estrus usually indicates that the female cannot achieve an ovulatory surge of LH. The ovaries of such females are polyfollicular and contain no corpora lutea (20). Serum estradiol concentrations are appreciable and progesterone concentration is minimal in the CE female (21). A pattern of constant vaginal estrus may be induced by compounds that interfere with the neuroendocrine control of ovulation (22). The delayed anovulatory syndrome (23) is typified by a constant estrus pattern that develops after puberty following neonatal treatment with estradiol, diethylstilbestrol or a variety of estrogenic pesticides (24).

Interestingly, the constant estrous female may be sexually receptive and ovulation may be induced upon mating (25), but the fertility of such matings has not been thoroughly evaluated. In our laboratory, we found that although young-adult, spontaneously constant estrous females rats did mate readily, pregnancy outcome was significantly reduced, in that only 25 % became

pregnant and those that did had reduced litter sizes (N=15, mean = 5.57 ± 1.43 vs. control N = 22, mean = 13.4 ± 0.9, Cooper unpublished).

Long-term exposure of adult female mice to estrogen has many deleterious effects on vaginal cytology, as well as neuroendocrine function (26). Chronic estradiol treatment of C57BL/6J female mice selectively impairs the ability of the vagina to produce cornified epithelial cells. Vaginas of female mice exposed for 3 to 5 months to either high or low levels of estradiol fail to cornify in response to new estradiol implants. Vaginal cornification was also significantly reduced in 23 month-old mice. These results suggest that both age and chronic exposure to estradiol impair estradiol-induced vaginal cornification.

Successful pregnancy in the female rat and mouse depends upon two sets of physiological events: (a) transport of gametes through the reproductive tract so that fertilization can be effected and (b) establishment of an appropriate hormonal environment (progestational state), through cervical stimulation, so that the fertilized egg can implant in the uterus and be maintained during subsequent gestation. Pseudopregnancy is actually an endocrine pregnancy that can be experimentally induced by stimulating the uterine cervix of the female (normally a consequence of male intromission behavior) on diestrus II, the day of vaginal proestrus or estrus (27). The pseudopregnant female's ovaries contain several prominent corpora lutea and the uterus is well- developed. The vaginal smear of such females is

predominantly leukocytic for 12-16 days.

Anestrus is indicated when the vaginal smear remains leukocytic indefinitely. The ovaries of the anestrus female are atrophic, with few primary follicles and an unstimulated uterus (20). Serum estradiol and progesterone are minimal. Anestrus, or prolonged vaginal diestrus, may be indicative of compounds that interfere with follicular development or deplete the pool of primordial follicles (28, 29).

The persistence of regular vaginal cycles following treatment does not necessarily indicate that the compound is not a reproductive toxicant, since such cycles may be anovulatory. Thus, the follicles may be luteinized without rupturing, such as those observed following treatment with anti-inflammatory agents (18). A compound may adversely affect the oocyte itself, the transport of the oocyte once it is released, or the processes involved in fertilization, implantation and pregnancy maintenance (30). Irregular cycles may reflect impaired ovulation, as delayed ovulation may extend the period of vaginal cornification (e.g., 2-3 days). Such cycles would be typical in animals exposed to anesthetics (i.e., phenobarbital or pentobarbital) or noradrenergic blocking compounds during the critical period for the neural trigger of the LH surge (12, 15, 31, 32).

References

1. C.R. Stockard, and G.N. Papanicolaou, Am. J. Anat. 22, 225 (1917).
2. E. Allen, Am. J. Anat. 30 297 (1922).
3. J. A. Long, and H. M. Evans, The oestrous cycle in the rat and its associated phenomena. Memoirs. University of California, 6, 1 (1922).
4. M. K. McClintock, In "Pheromones and Mammalian Reproduction", (J. G. Vandenbergh, ed.), p. 113, Academic Press, N. Y. (1983).
5. J.G. Vandenbergh, In "Pheromones and Mammalian Reproduction", (J. G. Vandenbergh, ed.), p. 95, Academic Press, N. Y. (1983).
6. R. Rugh, In "The Mouse. Its Reproduction and Development." Burgess Pub Co., Minneapolis MN (1968).
7. J.G. Vandenbergh, Endocrinology 84, 658 (1969).
8. A. K. Champlin, D. L. Dorr, and A. H. Gates, Biol Reprod 8, 491, (1973).
9. H. Underwood, J. M. Whitsett, and T. G. O'Brian, Biol. Reprod. 32, 947 (1985).
10. J. A. Elliott, In "Biological Clocks in Seasonal Reproductive Cycles", B. K. & D. E. Follett, eds.), p. 203, Academic Press, N.Y. (1981).
11. K. Hoffmann, J. Comp. Physiol. 148, 529 (1982).
12. J. W. Everett, "Neurobiology of Reproduction in the Female Rat."

Springer-Verlag, New York (1989).

13. L. C. Krey and J. W. Everett Endocrinology 93, 377 (1973).

14. S. V. Drouva, E. Laplante, and C. Kordon, Eur. J. Pharm. 81, 341 (1982).

15. J. M. Goldman, R. L. Cooper, T. L. Edwards, G. L. Rehnberg, W. K. McElroy, and J. F. Hein, Pharmacol. Toxicol. 68, 131 (1991).

16. R. L. Cooper, R. W. Chadwick, G. L. Rehnberg, J. M. Goldman, K. C. Booth, J. F. Hein, and W. K. McElroy, Toxicol. Appl. Pharmacol. 99, 384 (1989).

17. P. van der Schoot, J. Endocrinol. 69, 287 (1976).

18. R. F. Walker, L. W. Schwartz, and J. M. Manson Toxicol. Appl. Pharmacol. 94, 266 (1988).

19. A. M. Cummings and S. D. Perreault, Toxicol. Appl. Pharmacol. 102 110 (1990).

20. H. H. Huang, and J. Meites, Neuroendocrinology 17, 289 (1975).

21. H. H. Huang, R. W. Steger, J. F. Bruni, and J. Meites, Endocrinology 103, 1855 (1978).

22. R. F. Walker, R. L. Cooper, and P. S. Timiris, Endocrinology 107, 249 (1980).

23. R. A. Gorski, Endocrinology 82, 1001 (1968).

24. L.E. Gray, Jr. In "Aging and Environmental Toxicology: Biological and Behavioral Perspectives," (R.L. Cooper, J.M. Goldman and T.J. Harbin, eds.), p. 183. John Hopkins Univ. Press, Baltimore, (1991).

25. K. Brown-Grant, J. M. Davidson, and F. Grieg, Endocrinology 57, 7 (1973).

26. A. J. Adler, and J. F. Nelson, Biol. Reprod. 38, 175 (1988).

27. J. Terkel, J. A. Witcher, and N. T. Adler, Eur J. Pharm 81, 341 (1990).

28. J. J. Heindel, P. J. Thomford, and D. R. Mattison, In "Growth Factors and the Ovary," (A.N. Hirshfield, ed.), p 421. Plenum, New York, (1989).

29. R. L. Dobson, and J. S. Felton, Am. J. Ind. Med. 4, 175 (1983).

30. A. M. Cummings, Fund. Appl. Toxicol. 15, 571 (1990)

31. N. W. Fugo, and R. L. Butcher, Fertil. Steril. 17, 804 (1966).

32. P. F. Terranova, Biol Reprod 23, 92 (1980).

Figure Legends

Figure 1. Schematic of the rat four-day estrous cycle depicting relationship among vaginal cytology, serum estradiol, and serum LH (m = midnight).

Figure 2. Photomicrographs (100X) showing major changes in the vaginal smear during the estrous cycle in the rat. The absolute number of cells may vary from sample to sample. **A.** *Diestrus*, typically represented by many leukocytes which may or may not be mixed with varying numbers of larger cornified epithelial cells. **B.** *Proestrus*, is signalled by the appearance of rounded polynucleated epithelial cells which may initially occur as wispy or stringy aggregates (b1) and later as numerous clumps (b2). **C.** *Estrus*, represented by the predominance of cornified epithelial cells. **D.** *Metestrus*, represented by dispersed, round, non-nucleated cells. Leukocytes are frequently present during this period and show considerable variations in number.

Figure 3. Photomicrographs (100X) showing major changes in vaginal exfoliative cytology during the estrous cycle in the female house mouse. The lavage in the mouse is taken with a fire polished Pasteur pipette rather than an eye dropper in the rat. The absolute number of cells may vary from sample to sample, and a female may remain in phase of the cycle, usually diestrus, for several days. **A.** *Diestrus*, represented by leukocytes, often in large number, a

variable number nucleated epithelial cells and few to no cornified epithelial cells. **B.** *Proestrus*, represented by a variable number of leukocytes and a large number of nucleated epithelial cells with few to no cornified cells. **C.** *Estrus*, represented by the absence of leukocytes, few or no nucleated epithelial cells and many flat cornified epithelial cells. **D.** *Metestrus*, represented by the infiltration of leukocytes, little or few nucleated epithelial cells, and a variable number of cornified cells, often beginning to "roll".

Figure 4. Common technique used for vaginal lavation. An alternative method is to elevate the female's hindquarters by holding the base of her tail and lifting slightly.

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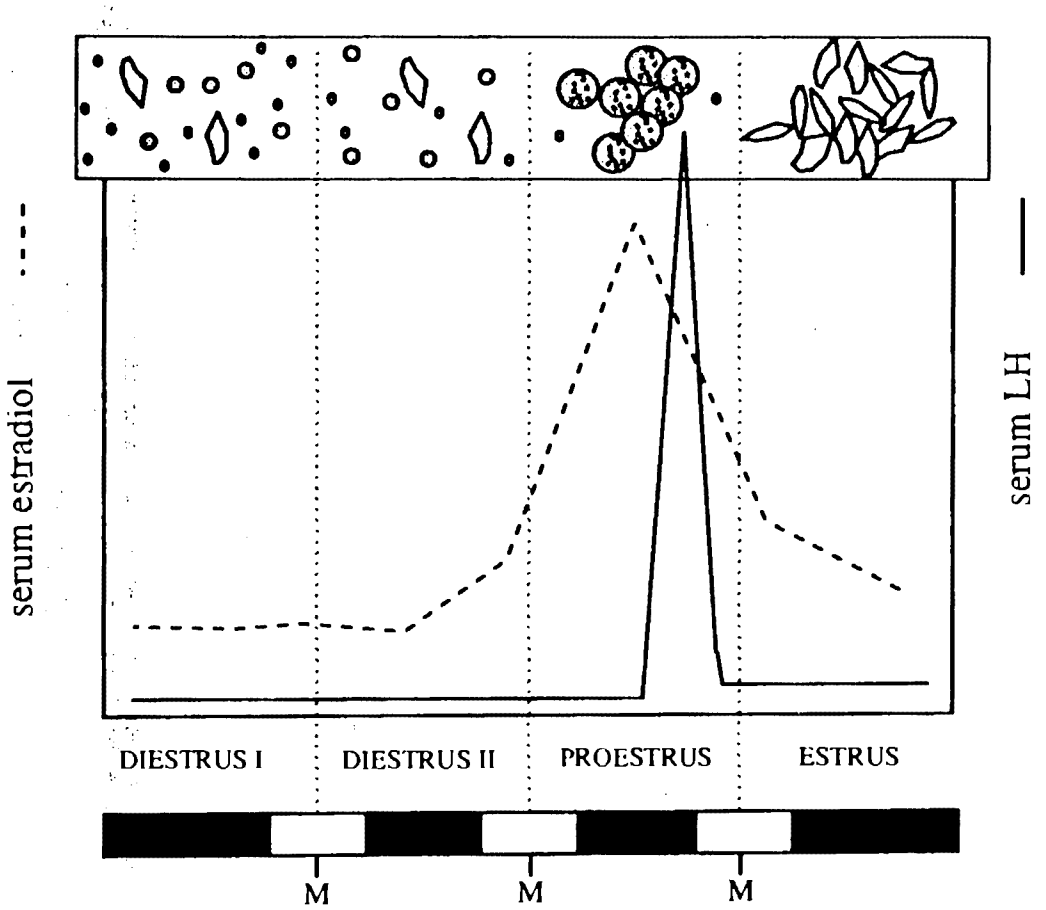
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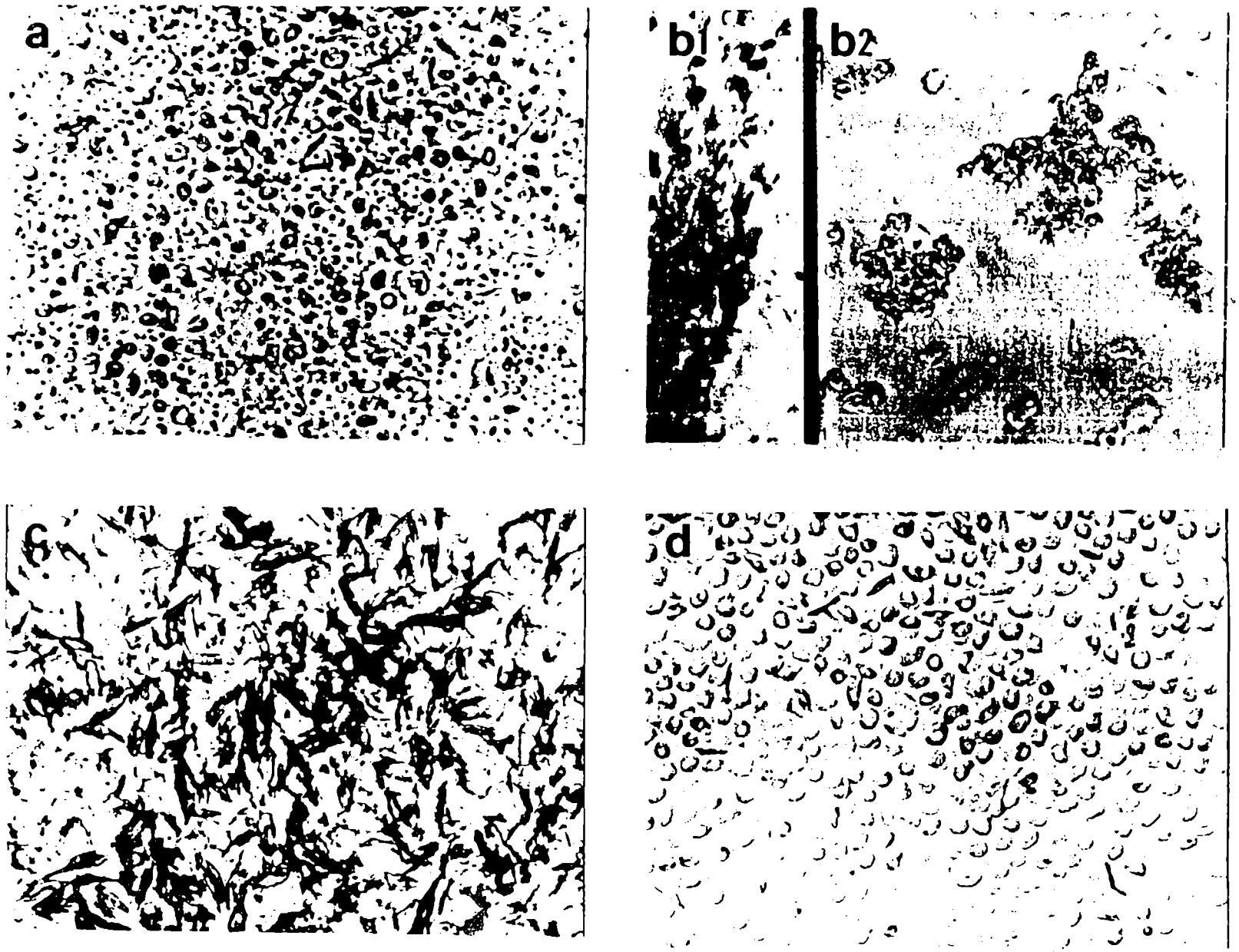
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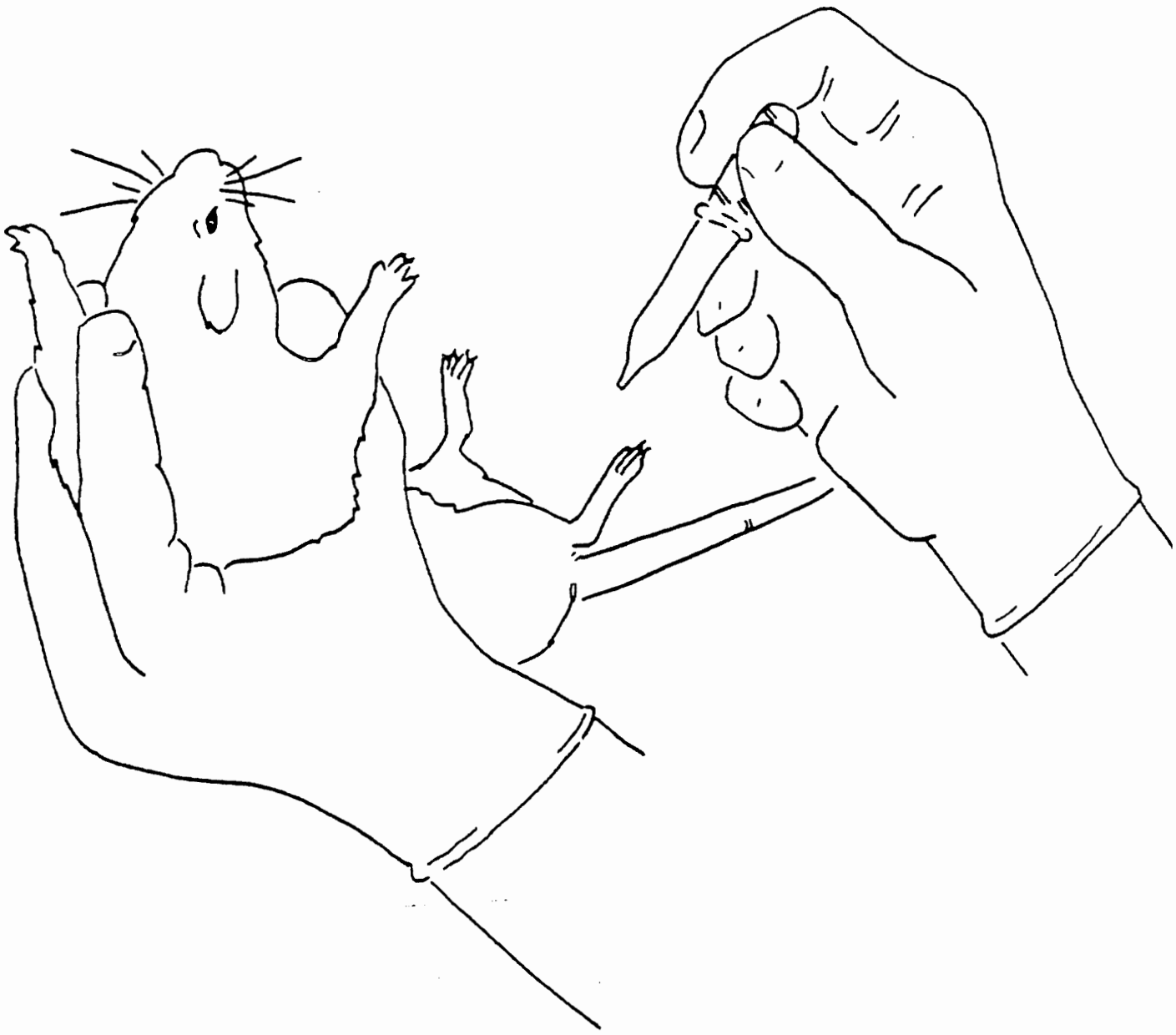

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16. ABSTRACT

Ovarian cyclicity in a number of laboratory species can be monitored easily and noninvasively by observing changes in the vaginal cytology. This chapter describes the techniques used to collect data in the laboratory rat and mouse and how to interpret the lavages as they are obtained. The relationship between the various cell types and the fluctuation in serum hormones as they exist over the cycle is described. Finally, there is a discussion of how to interpret various changes in vaginal smear patterns as they occur in response to toxicant treatment.

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