Chapter 9. Fetal Examinations and Vaginal Cytology

Specifications for the Conduct of Toxicity Studies by the Division of Translational Toxicology at the National Institute of Environmental Health Sciences

From: Roberts GK, Stout MD, editors. Specifications for the Conduct of Toxicity Studies by the Division of Translational Toxicology at the National Institute of Environmental Health Sciences. Research Triangle Park, NC: National Institute of Environmental Health Sciences; 2023.
https://doi.org/10.22427/NIEHS-00

9. Fetal Examinations and Vaginal Cytology

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Last Updated: March 2023

9.1. Prenatal Study Necropsy

9.1.1. Caesarian Section

Dams or does shall be euthanized before expected parturition (the morning of gestation day [GD] 21 for rats, GD 18 for mice, and GD 29 for rabbits) or as defined in the test article-specific Protocol Outline. Rats, mice, and rabbits shall be euthanized in a manner consistent with guidance provided in Chapter 6 (Laboratory Animal Medicine and Toxicology). Dams or does shall be examined and any abnormalities in the maternal thoracic or abdominal viscera recorded. Collection of tissues related to gross observations shall be conducted as described in the study protocol.

The uterus and ovaries shall be excised and weighed, and the number of corpora lutea in each ovary counted and recorded. The uterus of animals that do not appear pregnant shall be examined for nidations (implantation sites) by staining with 10% ammonium sulfide (or another suitable agent, such as Prussian blue).

The fetuses shall be removed from the uterus and their membranes as soon as possible after the dam is euthanized. The number and location of each implantation shall be recorded. The status of each implant site (live or dead fetus, early or late resorption) shall be recorded. The number of implantations shall be compared with the number of corpora lutea to determine pre-implantation loss. Corpora lutea shall be enumerated grossly at necropsy using a dissecting microscope.

- An early resorption is a conceptus where organogenesis has not been completed and is characterized by a grossly necrotic mass that has no recognizable fetal form; nidation sites (“pregnant by stain”) are also considered early resorptions.
- A late resorption is defined as one in which organogenesis has been completed and is characterized by a grossly necrotic but recognizable fetal form with visible placental remnants.
- A live fetus is pink in color and responds to stimulation.
- A dead fetus is a term fetus, often pale to tan in color and is not responsive to stimulation but does not demonstrate marked autolysis. Fetuses with marked autolysis are considered late resorptions.

Fetuses shall be separated from the placenta by cutting the umbilical cord, and any membranes shall be removed. The fetuses shall be blotted dry, individually identified, and individually weighed. Placentas shall be examined for abnormalities in appearance. Collection of tissue related to gross observations shall be conducted as described in the study protocol.

Dams euthanized moribund, delivered early, or found dead shall receive a gross necropsy, including an examination of the thoracic and abdominal viscera for evidence of dosing trauma, toxicity, or gross lesions. The uterus shall be examined and stained, if necessary, to determine pregnancy status. The animal shall be discarded without further examination.

9.1.2. Fetal Exams

Euthanize fetuses prior to visceral examination (e.g., oral administration of sodium pentobarbital). Confirm death by the absence of respiratory movement, absence of response to external stimuli, and/or body color change. Each fetus shall receive an examination as described below. Examination findings shall be recorded in accordance with the computer data system library (or other COR-approved process).

For rodent and rabbit dose range-finding studies:

- Fetal weights (live fetuses only) shall be obtained, and external examinations shall be conducted.
- The external examination shall include all body surfaces (including orifices) and appendages, and the mouth shall be opened and examined for cleft palate.
- The sex of each fetus shall be determined by inspection of the anogenital area for rodents and internally for rabbits (internal confirmation for rodents when it is in doubt).

For definitive rodent and rabbit studies:

- All fetuses shall be subjected to external, visceral, and skeletal examinations.
- Visceral examinations shall be conducted under a dissecting microscope.
- The sex of each fetus shall be confirmed by internal examination of the gonads.
- Approximately 50% of each litter shall have the head removed just below the exoccipitals, leaving the cervical region intact. The isolated head should be fixed in Bouin's solution (rodents) and subjected to examination by serial razor blade sectioning. Alternative methods for fixation may be required by the sponsor or proposed by the testing laboratory for review and approval by the contracting officer's representative (COR). Heads from all rabbit fetuses will be examined by sectioning the skull and brain at the level of the frontal-parietal suture and examining the brain in situ.
- The remaining carcass shall be processed for skeletal examination (along with the other half of the litter with skulls attached), and fetuses shall be individually identified in a manner (e.g., tag, container coding) so they can be identified throughout processing, reading, and archiving.
- All skeletons shall be double-stained with Alcian blue and alizarin red (Ovchinnikov 2009) and stored in an appropriate manner (the staining procedure shall be optimized for a respective lab and the methodology approved by the COR).
- Fetal skeletal exams shall be conducted under a dissecting microscope.

The external examination shall include fetal weight (live fetuses only) and all body surfaces (including orifices) and appendages, and the mouth shall be opened and examined for cleft palate.

The visceral examination and evaluation shall include an assessment of color, size, shape, and position of:

- The abdominal viscera, including the organs of the digestive system (intestines, stomach, pancreas, spleen, liver [and gall bladder, except rats]), urinary system (kidneys, ureters, urinary bladder), adrenals, and reproductive system (for the female: ovaries and uterus; for the male: testes and epididymides) (kidneys shall be sectioned at the hilus and the renal papilla examined)
- The following thoracic viscera: thyroid, trachea, esophagus, thymus, lungs, diaphragm, and greater vessels of the heart (right and left subclavians and carotids, innominate, pulmonary arch, pulmonary artery, vena cava, aortic arch, aorta, and the ductus arteriosus)
- The following aspects of the heart and vessels:
    - the shape and position (with any abnormalities recorded)
    - the aorta, pulmonary artery, descending aorta, innominate (brachiocephalic), subclavian, common carotid arteries, and the ductus arteriosus
    - the internal anatomy of the heart, including the aortic, tricuspid, mitral, and pulmonic valves, as well as the interventricular septum

The skeletal examination shall include:

- The bones of the skull from approximately 50% of the fetuses: premaxillae, maxillae, nasals, frontals, parietals, interparietal, supraoccipital, exoccipitals, zygomatics, squamosals, and mandibles
- The axial skeleton: vertebrae (centra and arches), sternebrae, and ribs
- The pectoral girdle and pelvic girdle:
    - dorsal scapulae and clavicles
    - ilia, ischia, and pubis
- The forelimbs and hindlimbs:
    - humerus, radius, ulna, carpals, metacarpals, phalanges
    - femur, tibia, fibula, tarsals, metatarsals, phalanges

For fetal head examinations from approximately 50% of the fetuses (Thompson 1967):

- A ventrodorsal section (mouth and bisecting both ears) shall be made and the following tissues examined: tongue, palate, upper lips, and lower jaw.
- A frontal section anterior to the eyes shall be made and the following tissues examined: nasal septum, nasal sinuses, and palate.
- A frontal section that bisects the eyes shall be made and the following tissues examined: olfactory lobes of the brain, posterior areas of the nasal septum, nasal sinus

(nasopharyngeal cavity), palate, and fetal eye (including optic cup, retina, vitreous chamber, lens, and cornea).

- A frontal section posterior to the eyes (middle of the parietals) shall be made and the cerebral hemispheres (including the third and lateral ventricles) examined.
- Sections shall be retained in a manner in which each fetal head may be reassessed.

During the fetal examinations, all unusual (notable) observations shall be recorded using a sponsor-approved data collection system. The observations subsequently shall be deemed "malformations" or "variations," as per established criteria and designations [consistent with Makris et al. (2009)]. Historical control data are essential (along with concurrent controls) to determine the designation and occurrence of the findings in the context of normal background. In general, malformations are considered incompatible with—or severely detrimental to—postnatal survival (e.g., ventricular septal defect, exencephaly, diaphragmatic hernia). Variations are nonlethal and not considered detrimental to postnatal survival (e.g., reduced ossification of fore- and hind paws).

9.2. Vaginal Cytology Evaluation

9.2.1. Background

Many studies performed serve to identify target organs for toxicants. An important part of this strategy is evaluating the reproductive system. Microscopic evaluation of the types of cells present in vaginal smears has long been used to document the stages of the estrous cycle in laboratory rats and mice and as an index of the functional status of the hypothalamic-pituitary-ovarian axis. As such, assessment of the estrous cycle has been used both as a principal measure in determinations of reproductive cyclicity and as an ancillary test in reproductive toxicological studies (Goldman et al. 2007). Estrous cyclicity is a proven measure of female fecundity; prolonged cycles correlate with reduced litter size (Chapin et al. 1997).

Other female reproductive endpoints, such as reproductive organ weights, fluctuate with the estrous cycle stage; thus, weights are a less-than-useful endpoint for study-day-driven necropsies as females will be scattered through the cycle and weight variances will be high. Additionally, ovarian follicle counts, while specific, are a poor use of resources when exposure occurs in adulthood because (a) a small proportion of compounds has been shown to affect this parameter and (b) the data are exceedingly tedious and laborious to collect.

This relationship between estrous cycle stage and vaginal luminal cells (in guinea pigs) was first reported by Stockard and Papanicolaou (1917). Later, Long and Evans (1922) published on the vaginal cytology of rats and Allen (1922) on the vaginal cytology of mice. Much of the information regarding the evaluation of vaginal cytologies in rats and mice can also be found in the recent publication by Cora et al. (2015).

A detailed description of techniques to be used by the sponsor-designated laboratories to conduct vaginal cytology evaluations in toxicity studies properly and uniformly is provided in this document. The program COR for the toxicology testing laboratory will designate the dose levels for these studies. For vaginal cytology, 10 female rats or mice in each of three of the five dose groups, plus the control group(s), will be used. Specific selection of the three dose groups will be

based on the results obtained during the first 70 days of the subchronic studies. The objective is to select doses that are not causing overt toxic effects (e.g., mortality, depressed weight gain).

9.2.2. Evaluation in Mice and Rats

The following section is adapted from Cora et al. (2015).

9.2.2.1. The Estrous Cycle

The typical estrous cycle in rats and mice lasts 4 to 5 days, but 6-day cycles are encountered occasionally. Many environmental factors influence cycle length and include the light:dark cycle, age, noise, stress, social interactions (e.g., group housing), and whether a male rat or mouse are in the room. The estrous cycle consists of the four consecutive stages called proestrus (P), estrus (E), metestrus (M), and diestrus (D).

9.2.2.2. Cells of the Rat and Mouse Estrous Cycle

The stages of the estrous cycle are identified by the absence, presence, or proportion of the four cell types (Figure 9-1) described below. The relative changes in the cells present directly reflect the circulating levels of estrogen and progesterone secreted by the ovaries. Cell density and the arrangement of the cells in the smear also might aid in identifying the stages. The following descriptions of the cell types relate to dry-fixed smear preparations stained with a Romanowsky-type stain (e.g., Wright-Giemsa, modified Wright's) or Toluidine blue.

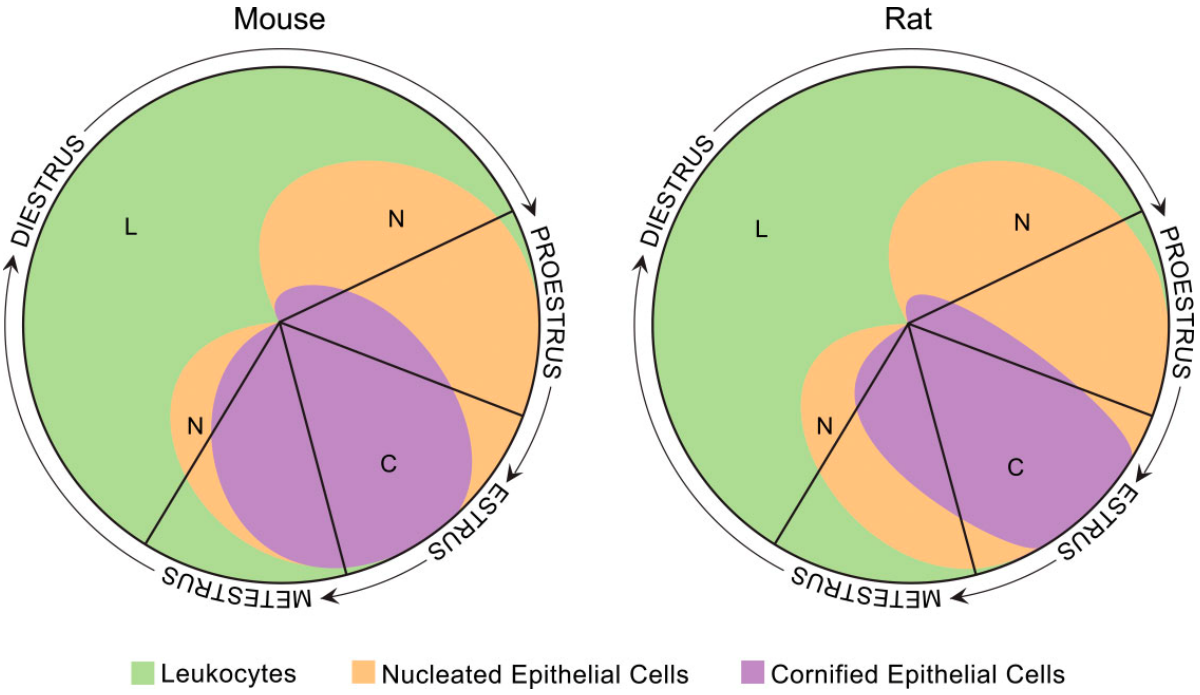

Figure 9-1. Estrous Cycle Wheels

These diagrams are visual representations of the cell types and relative proportion of each cell type present during the four stages of the estrous cycle in the mouse and rat. The size of each quadrant does not directly correlate to the length of each stage. From Cora et al. (2015); adapted from Byers et al. (2012)

Neutrophils (Figure 9-2). Neutrophils are also known as leukocytes or polymorphonuclear cells. These cells are very small and round and possess a multi-lobulated nucleus. During processing,

they can sometimes condense or "ball up," and have the appearance of a dark round dot. On higher power and with scanning of the smear, condensed neutrophils can usually be identified as such. Neutrophils are also relatively delicate and can partially rupture during collection or processing. The evaluator should be familiar with the appearance of condensed or ruptured neutrophils for accurate interpretation of vaginal smears.

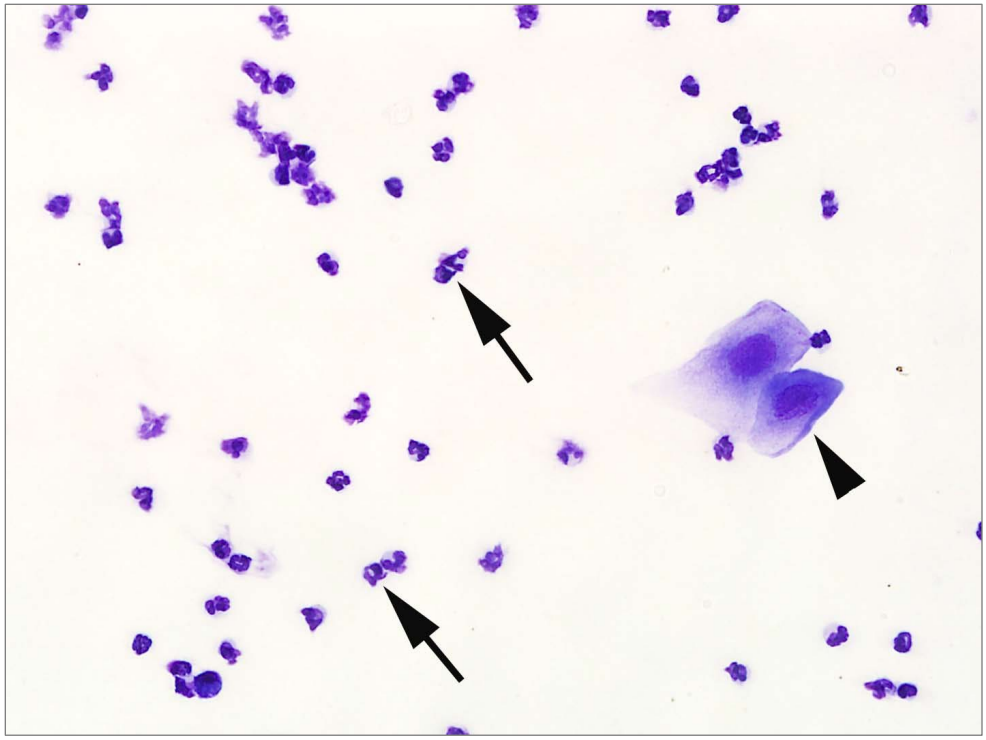

Figure 9-2. Appearance of Neutrophils in Vaginal Smears

Numerous neutrophils (arrows) are present. Note their small size in comparison with the large nucleated epithelial cells (arrowhead).

Small Nucleated Epithelial Cells (Figure 9-3). These cells are small and round to oval and have a round nucleus and blue cytoplasm. They can stain very darkly, precluding visualization of the nucleus. Small epithelial cells of proestrus may contain small cytoplasmic vacuoles.

Large Nucleated Epithelial Cells (Figure 9-2, Figure 9-4). These cells are round to polygonal and have smooth, jagged, or irregular borders. They possess moderate or abundant amounts of blue cytoplasm. Their nuclei could be intact, degenerate, or pyknotic. Large epithelial cells can have some degree of keratinization.

Anucleated Keratinized Epithelial Cells (Figure 9-4). Anucleated epithelial cells are aged cells with abundant blue to sky-blue cytoplasm and jagged or angular edges. As the name infers, they lack a nucleus but can sometimes contain a pale, round area where a nucleus once existed (a ghost nucleus).

9.2.2.3. Cytology of the Estrous Cycle

The cellular composition of the four basic stages of the estrous cycle is described below. Most smears can be evaluated with a 10X objective, but a 20X or 40X objective could be needed (for

example, to verify the presence of neutrophils). Sole use of higher objectives is not recommended because they prevent one from appreciating the overall impression of the smear. It is important to evaluate the whole smear because cell types and numbers can vary throughout the sample.

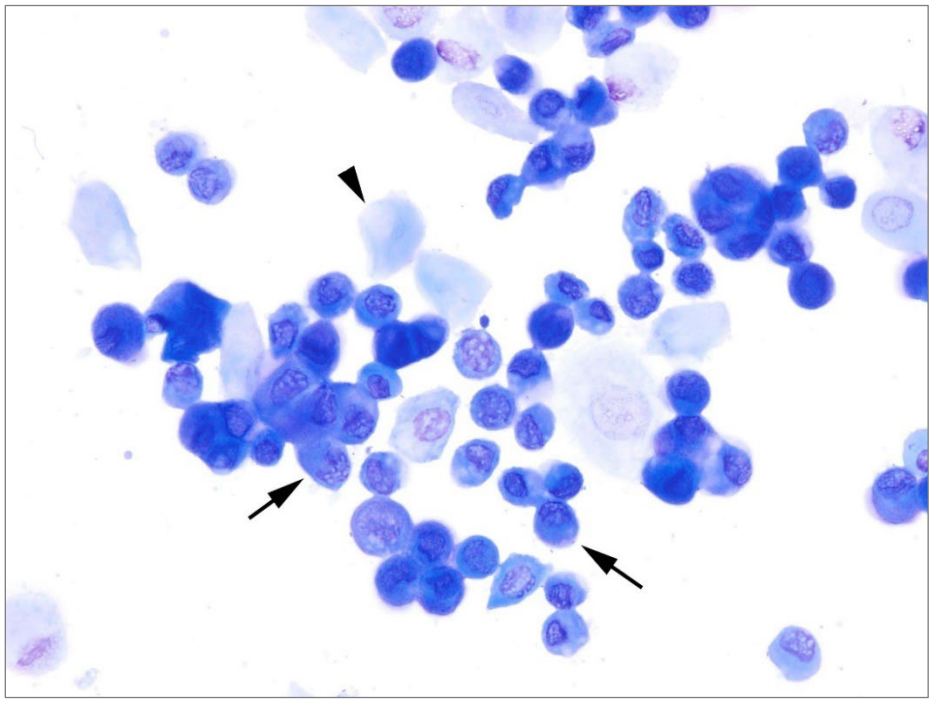

Figure 9-3. Appearance of Various Epithelial Cells in Vaginal Smears, Proestrus

Small nucleated epithelial cells (arrows) with a few anucleated epithelial cells (arrowhead) from a proestrus vaginal smear of a rat.

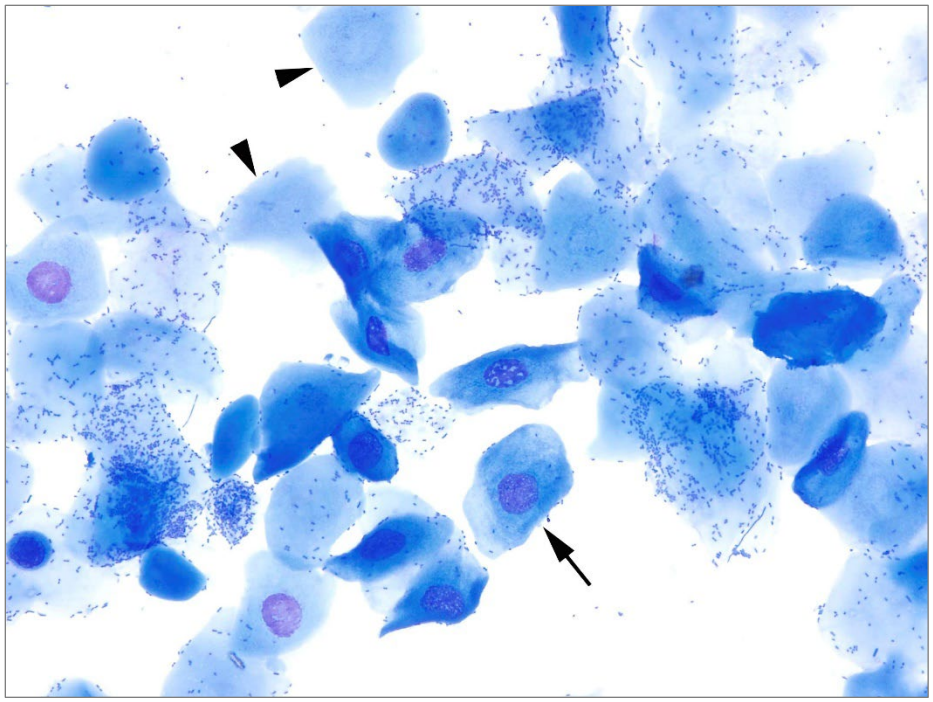

Figure 9-4. Appearance of Various Epithelial Cells in Vaginal Smears, Late Estrus

Large nucleated epithelial cells (arrow) and nucleated epithelial cells (arrowheads) from a vaginal smear of a rat in late estrus.

Evaluating all the collected days of an individual before making a diagnosis for each individual day can be important because it gives one a basic impression of how the rat or mouse is cycling and helps put each day into context. Care should be taken not to introduce bias and misidentify an abnormal stage as normal.

Although **relative** cell density can aid in defining a stage, it should **not** be used in isolation because it can vary independent of the actual stage of the estrous cycle. Collection technique, compliance of the rat or mouse during collection, and preparing more than one smear for each collection day can all affect the cell density of the smear.

The estrous cycle is a dynamic process with stages that are not all the same length—some lasting <24 hours. As such, some stages might be "missed" when sample collection occurs at the typical interval of every 24 hours.

One general approach to staging vaginal smears is to first assess the presence of neutrophils. Are there a good number of them or just an occasional one? In general, if neutrophils are a dominant feature or consistently observed, the stage is either metestrus or diestrus; if neutrophils are rare to absent, the stage is proestrus or estrus.

Proestrus (Figure 9-3, Figure 9-5, Figure 9-6). Proestrus is a relatively short stage that averages 14 hours in rats and <24 hours in mice. It is characterized by the presence of small nucleated epithelial cells of relatively uniform size and appearance. These cells can sometimes stain deeply basophilic. Other times, the small epithelial cells can take on a delicate or wispy appearance, especially in low-cellularity smears. The small epithelial cells can be observed in cohesive clusters (so-called "grape" clusters), sheets, or strands. **However, the observation of cohesive**

clusters, sheets, or strands should not be considered a prerequisite in the determination of proestrus as they might not always be visible, especially in low-cellularity samples. Neutrophils are usually not observed but can be found in low numbers in early proestrus as the rodent would have recently transitioned out of diestrus. Low numbers of large nucleated and anucleated epithelial cells might also be visible throughout proestrus, and, as the cycle approaches estrus, anucleated keratinized cells will become more abundant. The presence of low numbers of neutrophils, large epithelial cells, or anucleated epithelial cells does not preclude the recording of a proestrus stage when the predominating feature of the smear is the observation of high numbers of small epithelial cells.

Estrus. Estrus averages between 24 and 48 hours in rats and 12 and 48 hours in mice. Keratinized anucleated epithelial cells heavily predominate during estrus. Numerous bacteria might be adhered to the cells or scattered throughout the background of the smear. Low numbers of nucleated epithelial cells might be observed throughout estrus. Neutrophils are absent, although rare-to-occasional neutrophils might be seen toward the end of estrus as the rodent begins to transition to metestrus; once neutrophils are consistently visible throughout, the smear should be interpreted as metestrus.

Although consisting mostly of anucleated epithelial cells, the first and second “phases” of estrus differ in their appearance and also differ between the two species.

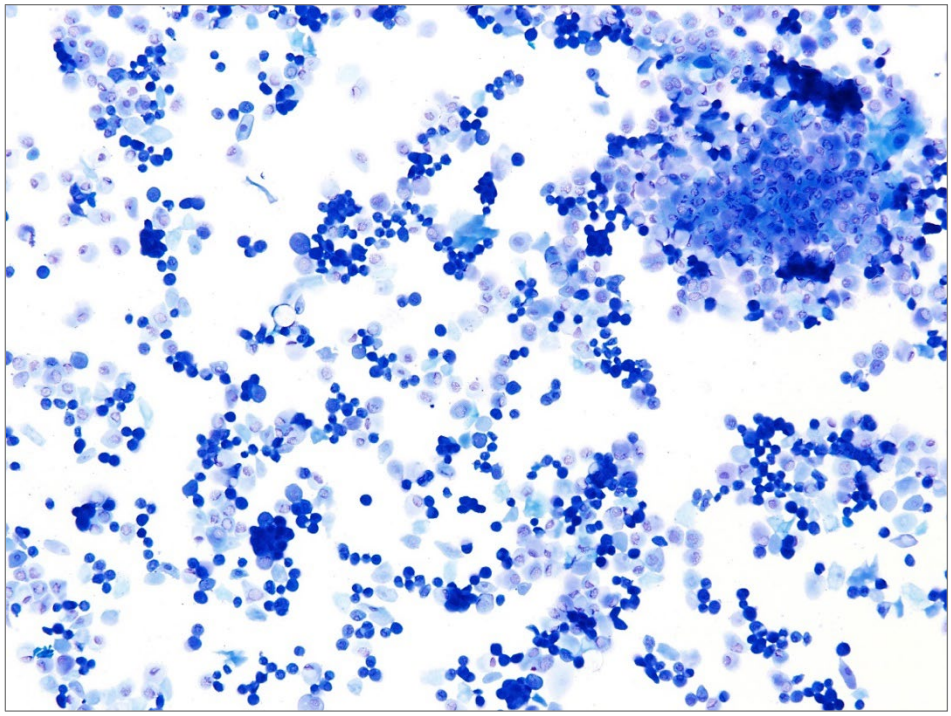

Figure 9-5. Proestrus: Example 1

Small, round nucleated epithelial cells, sometimes in clumps, predominate. No neutrophils are present.

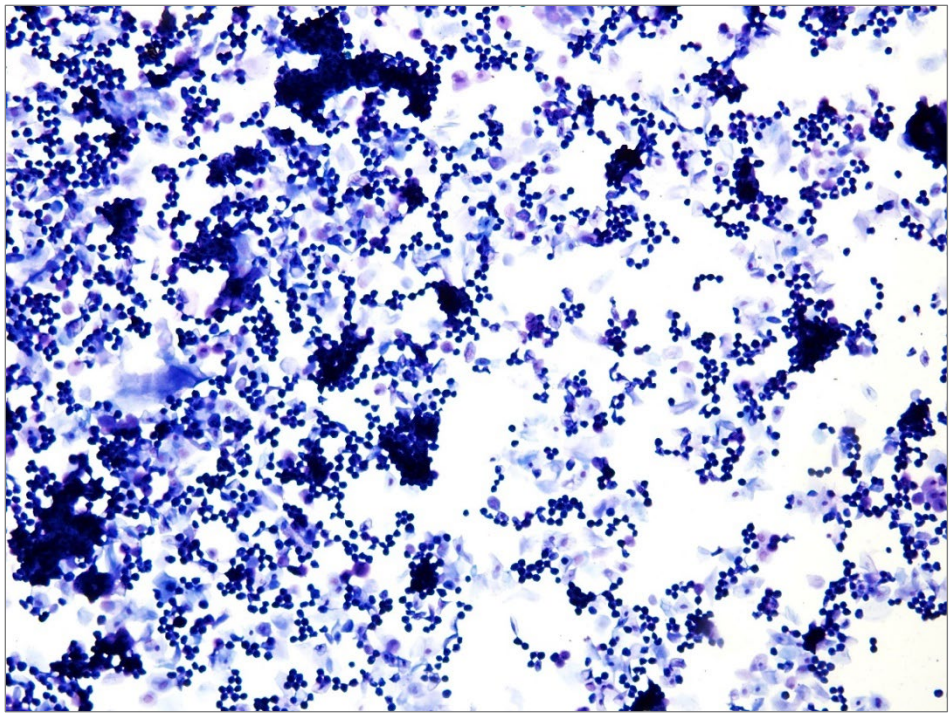

Figure 9-6. Proestrus: Example 2

Clumps of darkly stained, round, small epithelial cells predominate.

Rats (Figure 9-7, Figure 9-8, Figure 9-9, Figure 9-10). In rats, the so-called late estrous "phase" is distinctly different in appearance from the rest of the stage; however, it is a short phase, so it might not always be observed. Late estrus is characterized by the emergence of high numbers of small and large nucleated epithelial cells interspersed among the anucleated cells. The nucleated cells are smooth to irregular and might be round, oval, or spindle-shaped and sometimes stain deeply basophilic. The phase of late estrus should not be mistaken for proestrus. Evaluating the previous day's smear should help avoid confusion. Additionally, the nucleated cells of proestrus are generally more uniform in appearance.

Mice (Figure 9-11, Figure 9-12). When 2 days of estrus are observed in mice, the two different "phases" of estrus are usually appreciated. At the start of estrus in mice, the anucleated epithelial cells are smaller and usually arranged in loose clusters or sheets reminiscent of proestrus. As estrus progresses, the anucleated epithelial cells become larger and are usually more evenly distributed. The cells might be in stacks or layers.

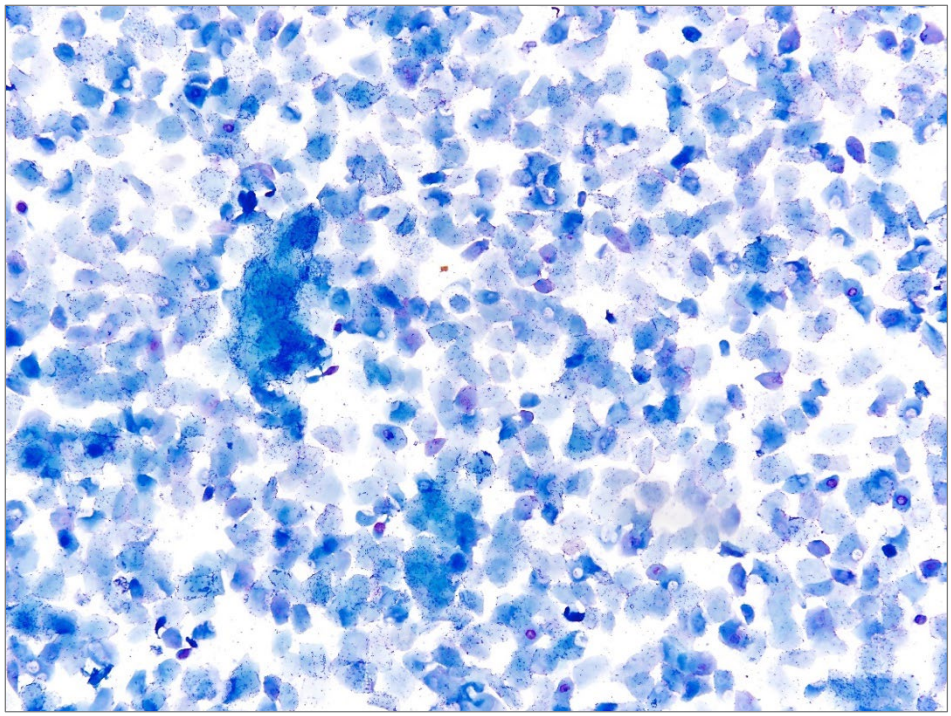

Figure 9-7. Estrus, Rat

Anucleated keratinized epithelial cells predominate.

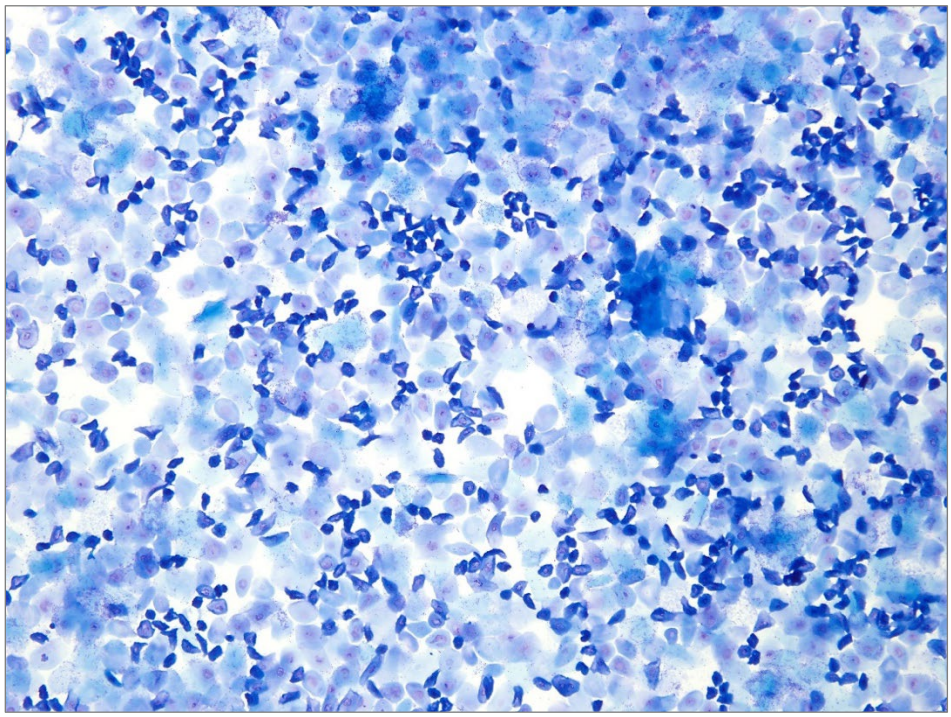

Figure 9-8. Late Estrus, Rat: Example 1

Darkly stained, oval to spindle-shaped nucleated epithelial cells are interspersed among the anucleated cells.

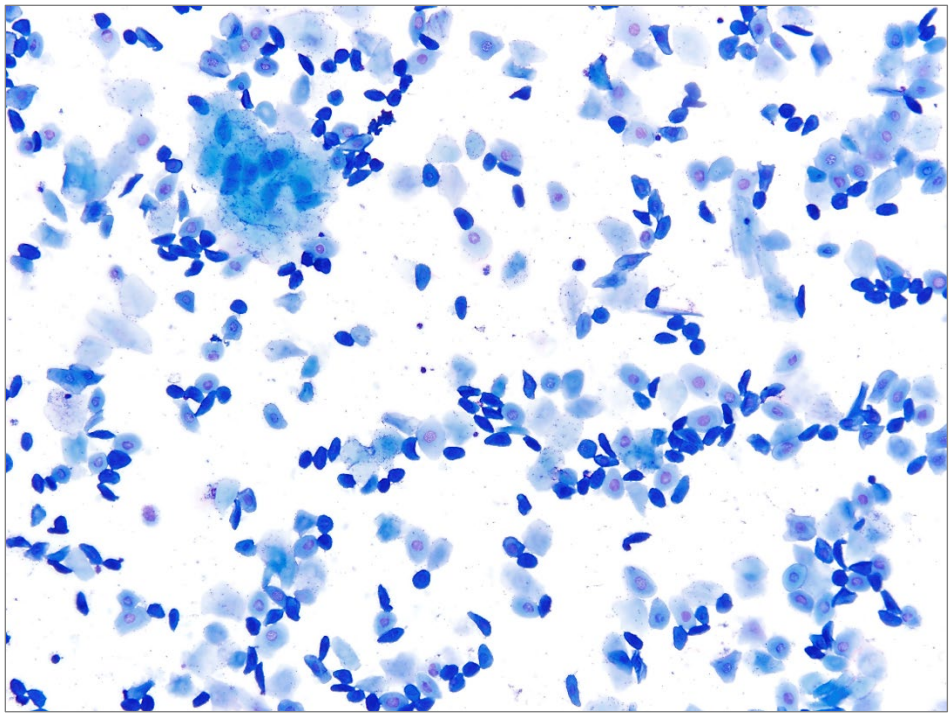

Figure 9-9. Late Estrus, Rat: Example 2

This image is another example of late estrus characterized by anucleated epithelial cells mixed with oval and spindle-shaped nucleated cells, some of which have stained deeply basophilic. Late estrus should not be mistaken for proestrus.

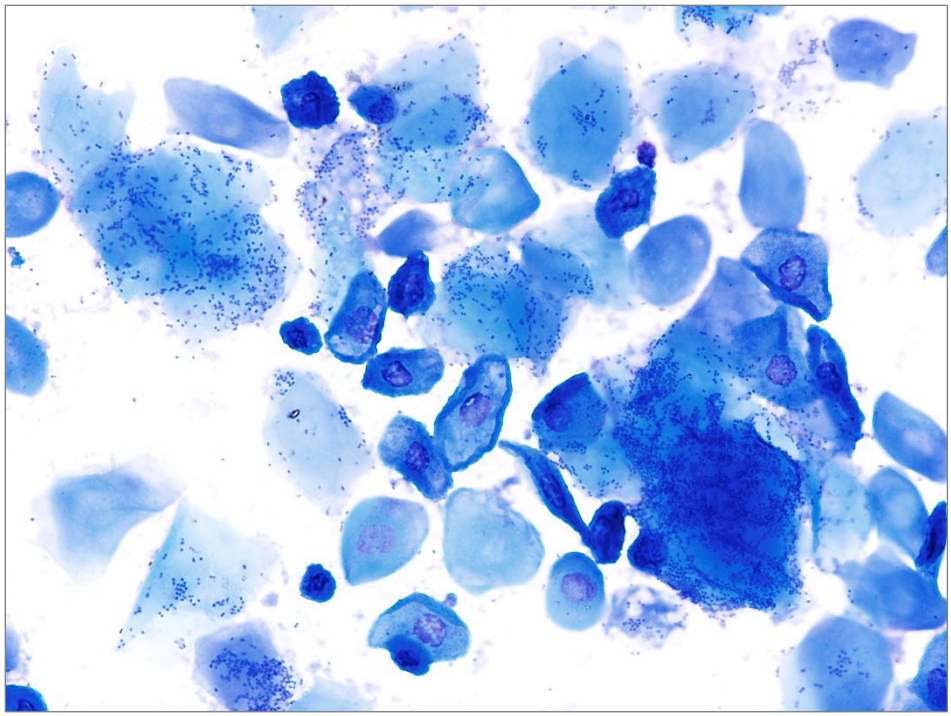

Figure 9-10. Late Estrus, Rat: Example 3

This image is a higher magnification of late estrus in a rat, showing the varying shapes of the nucleated cells. Also note the bacteria adhered to the cells and in the background, which is a normal finding in estrus.

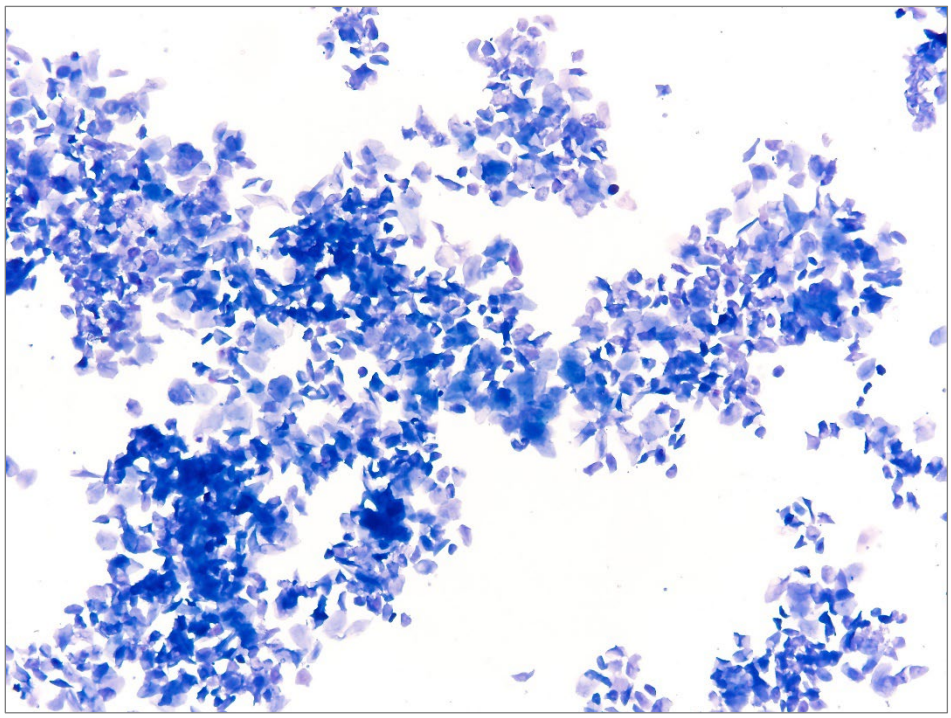

Figure 9-11. Estrus, Mouse: Example 1

In earlier estrus of the mouse, the anucleated cells are smaller and usually arranged in loose clusters reminiscent of proestrus.

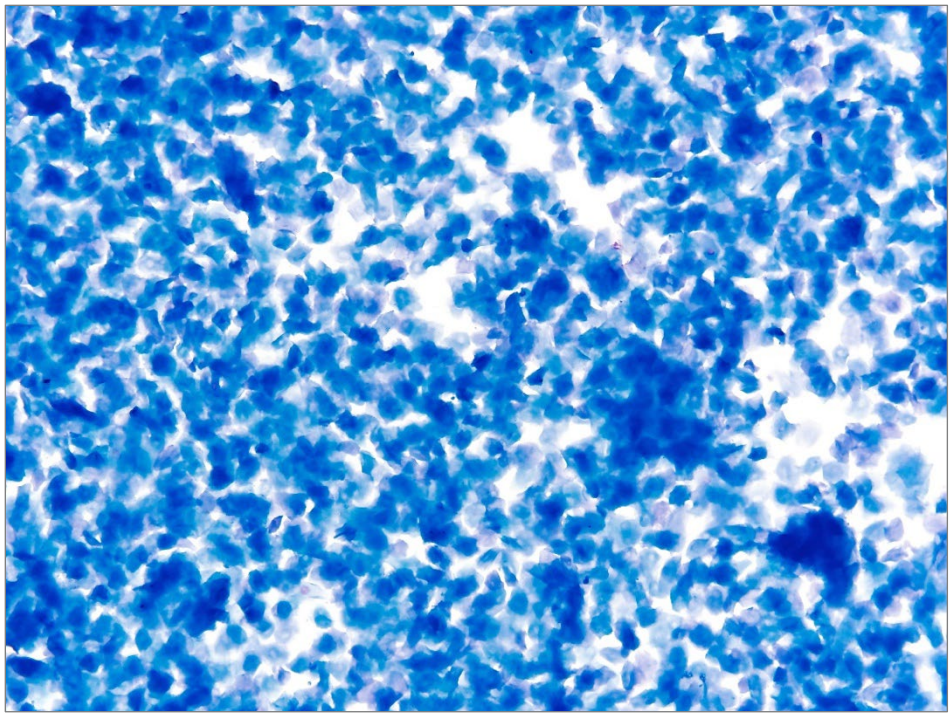

Figure 9-12. Estrus, Mouse: Example 2

As estrus progresses, the anucleated cells generally become larger, more evenly dispersed, and higher in numbers. Figure 9-11 and Figure 9-12 are 2 consecutive days of estrus from the same mouse.

Metestrus (Figure 9-13, Figure 9-14, Figure 9-15, Figure 9-16, Figure 9-17). Metestrus is a short stage in rats, lasting 6–8 hours. In mice, it can last as long as 24 hours, and, although infrequent, might be observed on two consecutive smears in mice. Metestrus is characterized by a combination of anucleated epithelial cells and neutrophils. In mice, rare-to-occasional nucleated epithelial cells might be observed. In rats, the nucleated epithelial cells of late estrus are present in moderate numbers throughout the stage.

In early metestrus, neutrophils are scattered among the epithelial cells and are sometimes found in clumps or tightly packed around the epithelial cells. At this point, the epithelial cell numbers predominate or are equal to the neutrophil numbers. As metestrus progresses, the neutrophil numbers greatly increase, resulting in a smear that is highly cellular; neutrophils can outnumber the epithelial cells by as much as 10-fold. Neutrophil and epithelial cell numbers decrease as the rodent transitions to diestrus.

The point at which metestrus ends and diestrus begins is not always obvious because they are defined by the same cell types and can have a similar appearance. Early and mid-metestrus are easily identified, however. If it is not clear whether a smear is late metestrus or early diestrus, it is best to be as consistent as possible and “err” on the side of diestrus. (There is minimal value in overly scrutinizing a late metestrus/early diestrus smear.)

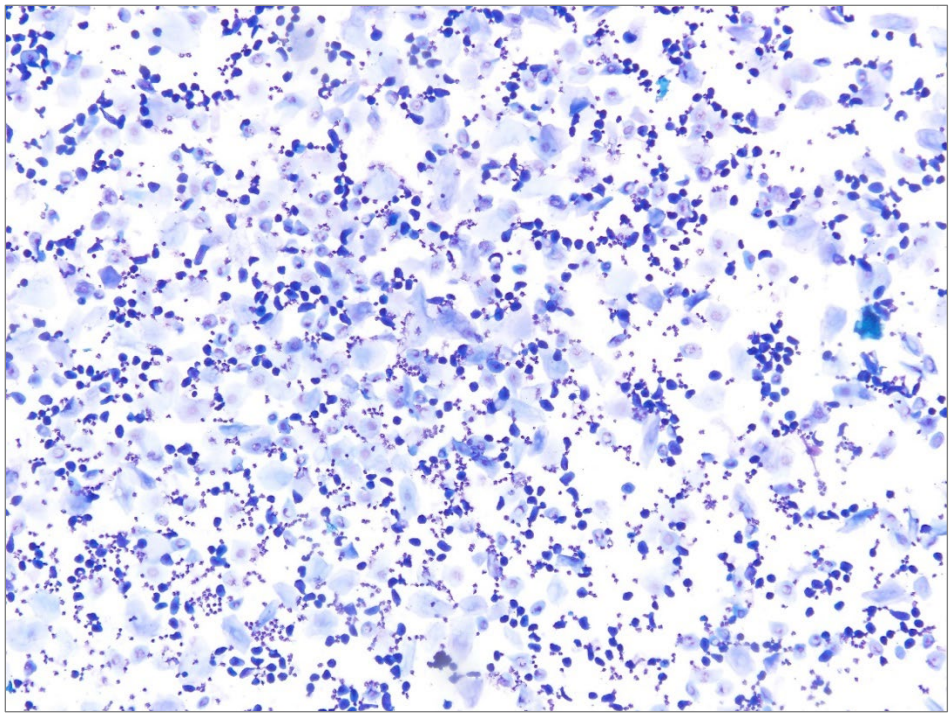

Figure 9-13. Metestrus, Rat: Example 1

Metestrus begins with the emergence of neutrophils interspersed or clumped among the nucleated and anucleated epithelial cells that are visible in late estrus.

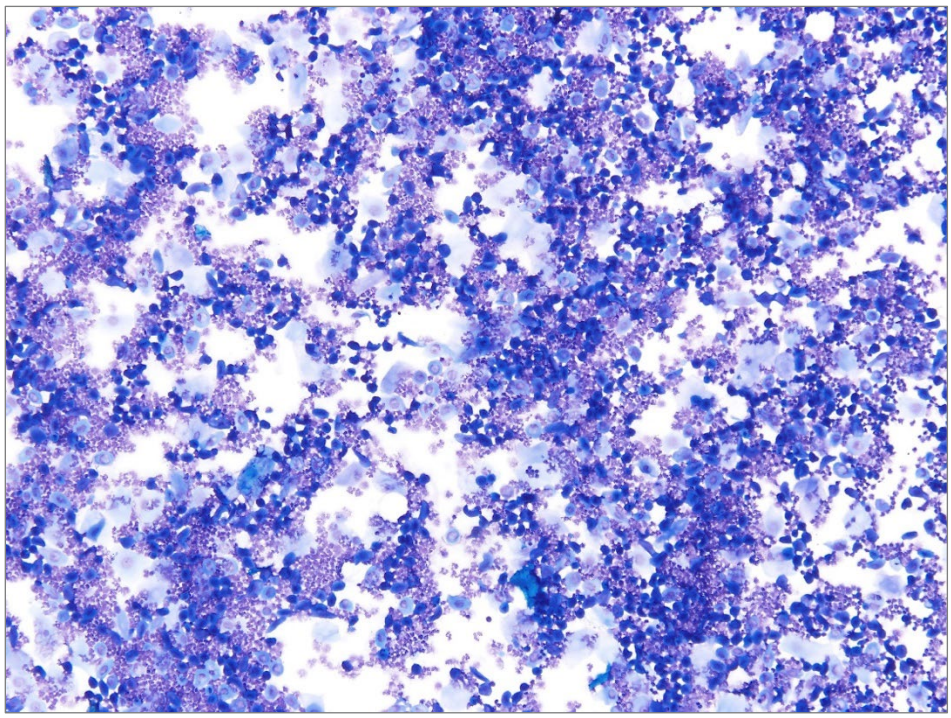

Figure 9-14. Metestrus, Rat: Example 2

As metestrus progresses, the neutrophil numbers increase substantially, resulting in a highly cellular smear.

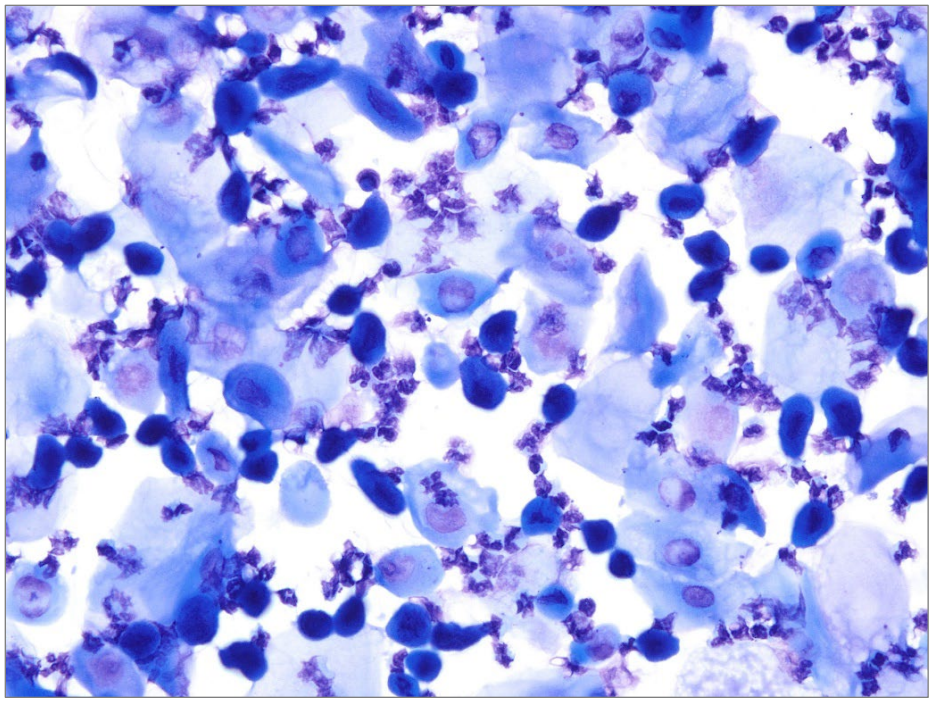

Figure 9-15. Metestrus, Rat: Example 3

This image is a higher magnification of rat metestrus, showing the mixture of anucleated epithelial cells, nucleated epithelial cells, and neutrophils.

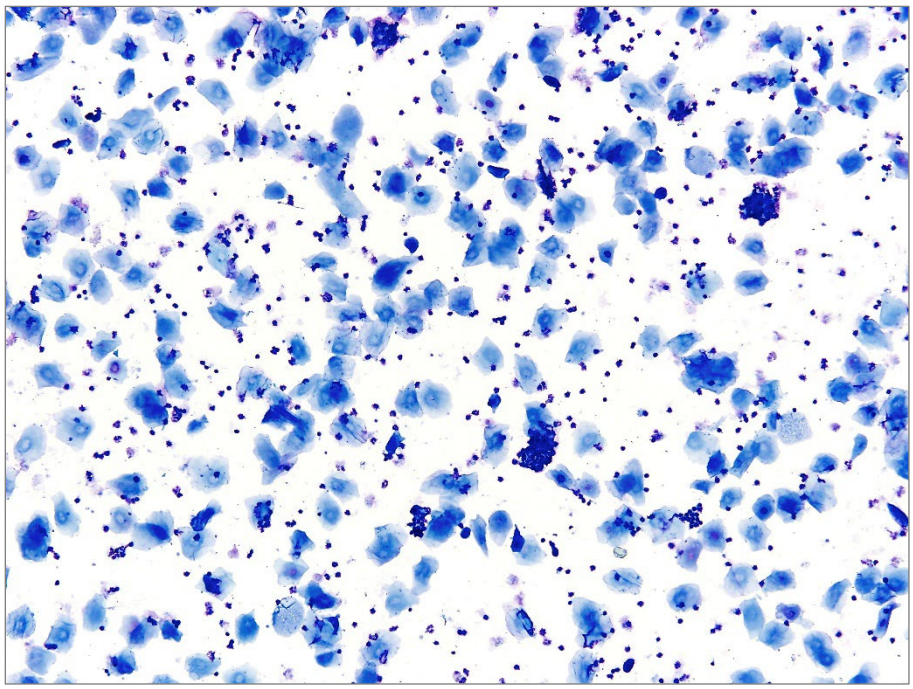

Figure 9-16. Metestrus, Mouse: Example 1

At the start of metestrus, neutrophils are interspersed or clumped around the anucleated epithelial cells. Note that metestrus differs between the mouse and rat in that rats have much higher numbers of nucleated cells mixed among the neutrophils; nucleated cells are in very low numbers in mouse metestrus.

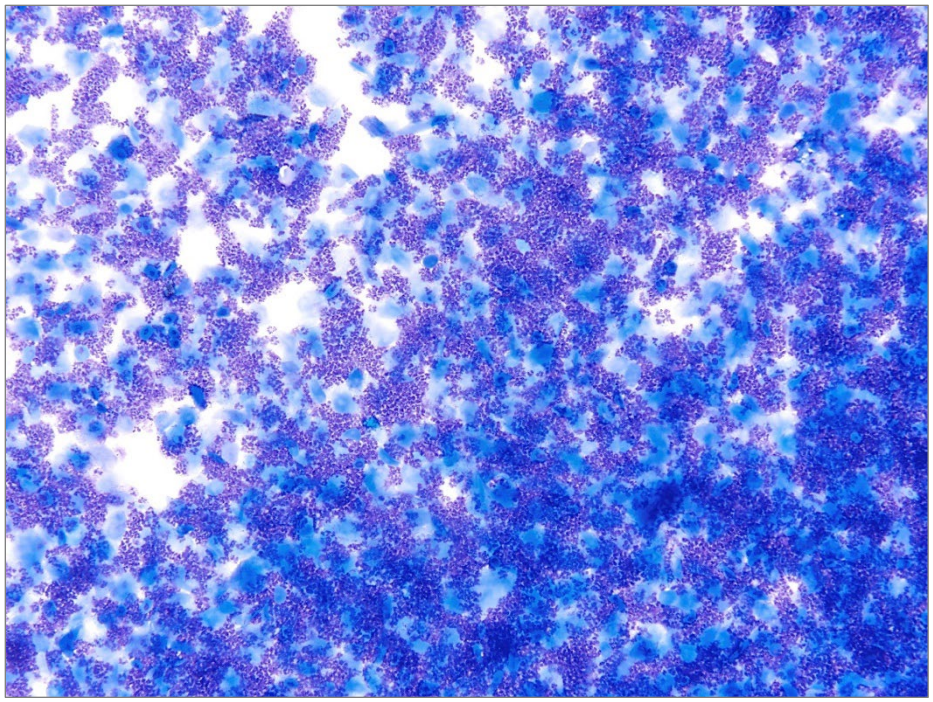

Figure 9-17. Metestrus, Mouse: Example 2

As metestrus progresses, the neutrophils become very high in number, resulting in a high-cellularity sample.

Diestrus (Figure 9-18, Figure 9-19, Figure 9-20). Diestrus is the longest stage, averaging 48–72 hours in rats and mice. There is a substantial decrease in the numbers (although not necessarily an absence) of anucleated epithelial cells. The cellularity is moderate to low, with a combination of small and large epithelial cells, neutrophils, and low numbers of anucleated epithelial cells. Neutrophil numbers are usually higher than epithelial cell numbers, with some smears being solely neutrophilic. In early diestrus, neutrophils might be visible in small clumps.

It is not uncommon for diestrus smears to have a very low cellularity with just a scattering of cells. This is especially true for days 2 and 3. Toward the end of diestrus, epithelial cells might also be observed in small clumps, indicating proestrus the next day, but neutrophils will still be a predominant feature of the smear.

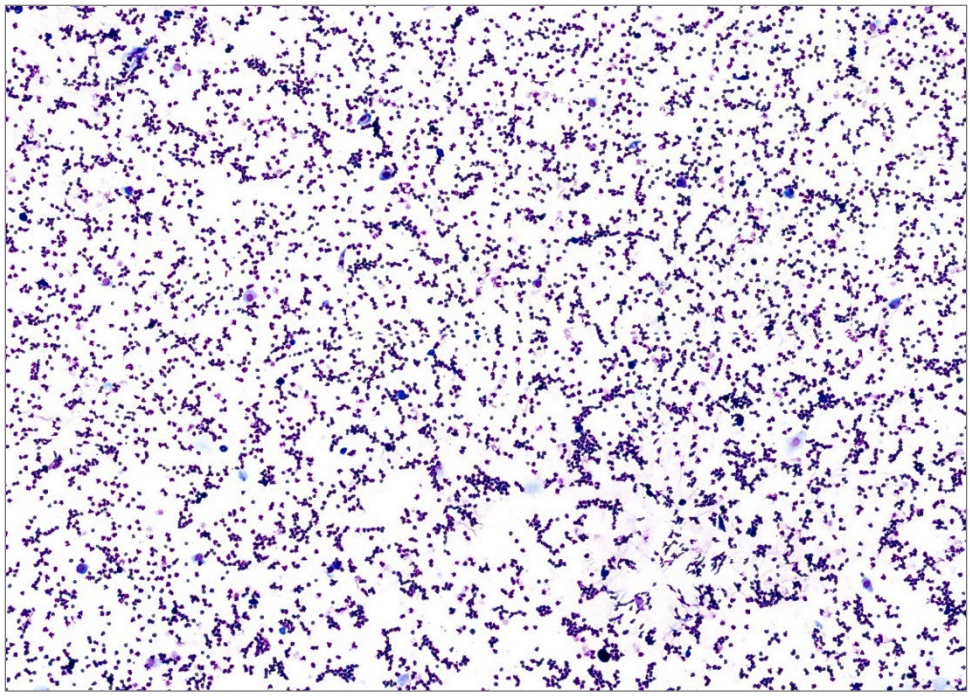

Figure 9-18. Diestrus: Example 1

Neutrophils predominate in this particular diestrus smear.

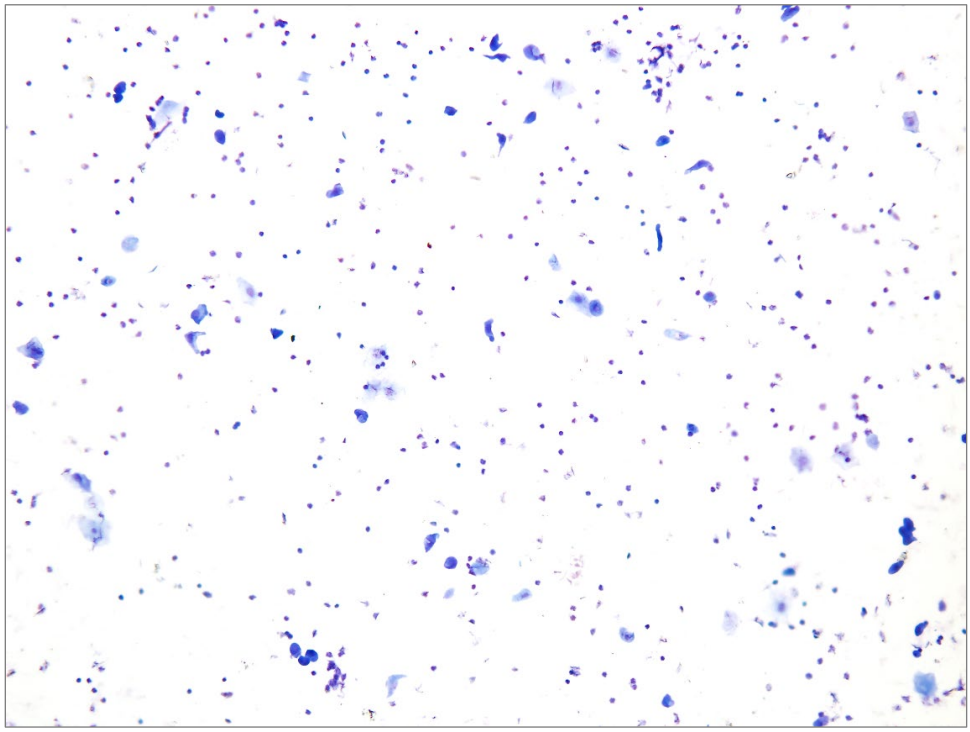

Figure 9-19. Diestrus: Example 2

Epithelial cells are interspersed among the neutrophils.

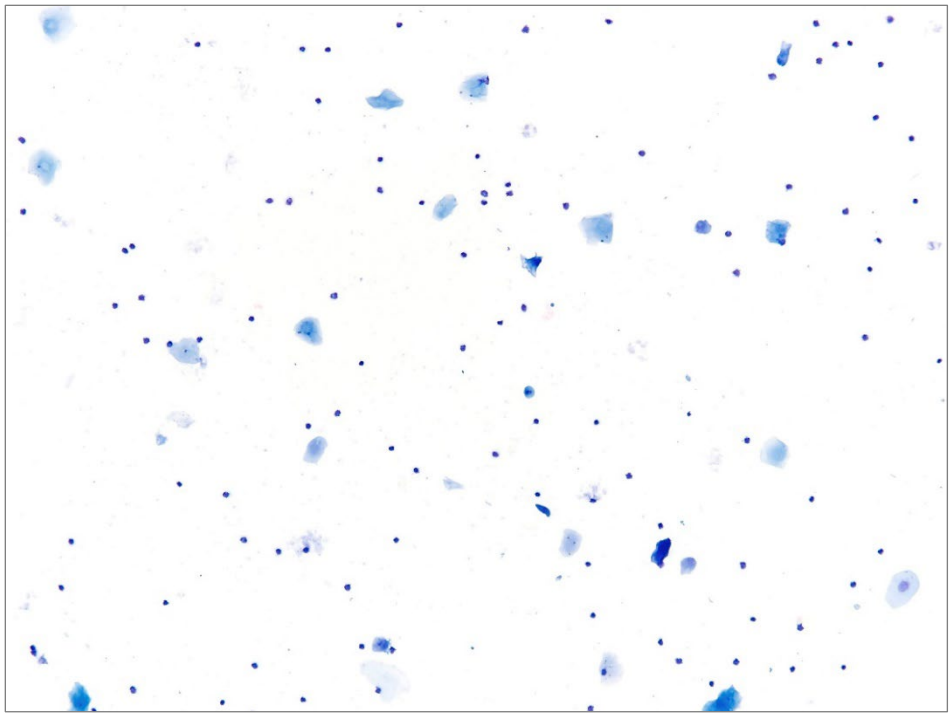

Figure 9-20. Diestrus: Example 3

Low-cellularity smear with a mixture of neutrophils and epithelial cells.

Transitional Stages (Figure 9-21). In general, classification of the individual stages, based on the criteria, is easily performed. It should be appreciated, however, that the estrous cycle is a process of constant change. As such, some samples may be collected at a time of transition (e.g., proestrus transitioning into estrus) and possess characteristics of both stages. In these situations, the recorded stage should be consistent with the most predominant feature(s) of that particular smear. For example, if the majority of cells in a smear transitioning from proestrus to estrus are small nucleated epithelial cells, the stage should be recorded as “P” for proestrus.

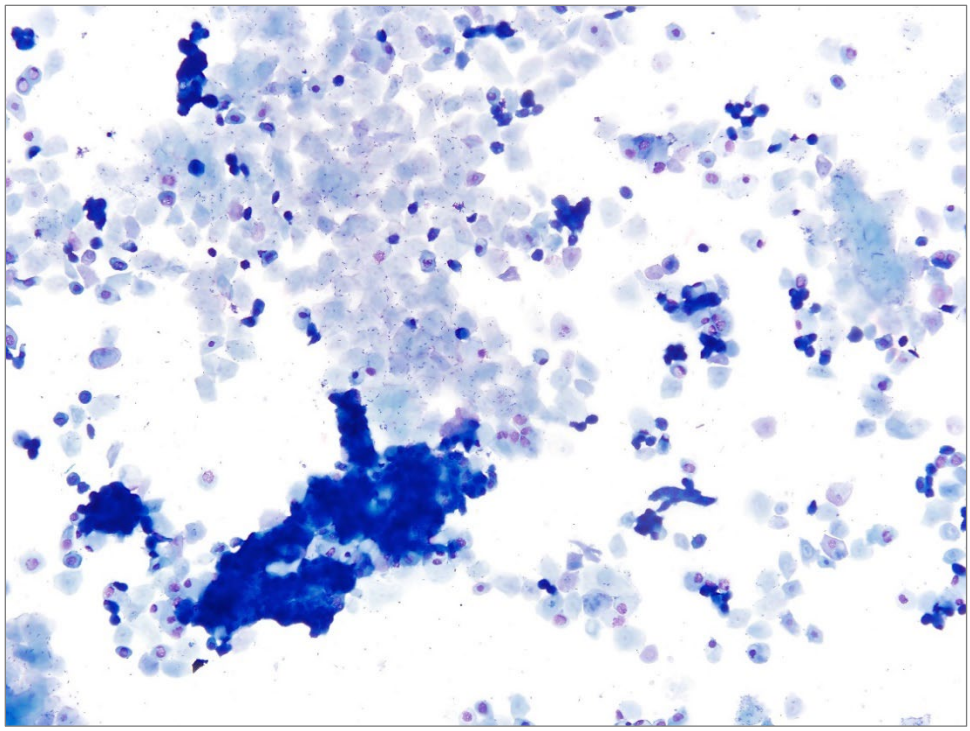

Figure 9-21. Proestrus to Estrous Transition, Rat

Many individual and clumps of small epithelial cells are interspersed with lesser numbers of lighter blue large nucleated and anucleated epithelial cells. A full evaluation suggests this smear was ultimately recorded as proestrus, as the small epithelial cells outnumbered the anucleated cells.

9.2.3. List of Suggested Materials for Vaginal Cytology Assays

These materials will be supplied by the toxicology testing laboratory:

- 0.9% saline solution or phosphate-buffered saline
- 100-slot slide boxes
- Beakers
- Coverslips (24 × 60 mm)
- Medicine droppers
- Microscope slide labels
- Parafilm
- Permount mounting medium and xylene
- Precleaned clear microscope slides (3 × 1 in.) with a frosted end
- Solvent-resistant permanent marker
- Trays

9.2.4. Procedure for Collecting and Preparing Vaginal Smears

Two vaginal smears shall be prepared from each animal between 8:00 and 10:00 a.m. each day during the final 16 consecutive days of the study. Duplicate smears may be used interchangeably during slide evaluation. The dose levels at which these are prepared shall be designated by the program COR for the testing laboratory.

Clear slides with a frosted end shall be used to make vaginal smears. All smears are made on the clear portion of the slide.

Using a solvent-resistant permanent marker, microscope slides are marked with a grid consisting of six squares per slide on the clear portion. The frosted end is marked with a permanent marker or pencil to indicate the species/animal number/slide (e.g., "01-M-001B" is sufficient at this stage because a label with complete information shall be applied later). The squares are labeled 1 through 6, 7 through 12, and 13 through 16 in the upper right corner. This technique is convenient as it allows the incorporation of 6 days of smears on 1 slide.

A 3–4 in. medicine dropper is moistened by aspirating a 0.9% saline or phosphate-buffered saline solution. A very small amount of the solution (up to the shoulder of the medicine dropper, approximately 0.2 mL) is left in the medicine dropper and then placed in the vagina (depending upon the strain, this depth should be approximately one-quarter to one-half of an inch for rats and less for mice) and the vaginal fluids are aspirated back and forth several times.

The contents of the medicine dropper are transferred onto the slide within the appropriate square for that animal-day. The use of excessive saline should be avoided to prevent flow onto surrounding squares. The slide should be checked for sufficient cell numbers (approximately 10–20/40× field) using a microscope. The slides are to be stored in slide folders in a dust-free atmosphere between collection dates.

After the 16th smear is taken and allowed to completely air-dry overnight, the slides can be stained with a Romanowsky-type stain (e.g., modified Wright's, Wright-Giemsa, or equivalent) by way of an automated slide stainer and according to manufacturer's instructions; this is the most consistent and efficient method.

Alternatively, slides may be stained manually with Diff-Quik, according to manufacturer's instructions, or with Toluidine blue as outlined in the following protocol:

- Preparation of 0.5% Toluidine blue stain: Mix 2.5 g of Toluidine blue with 500 mL of 20% ethanol and allow to sit for 1 hour before filtering through Whatman grade filter paper.
- Slides are loaded in glass racks and dipped in the following solutions:
    - 95% ethanol for 30 minutes
    - 80% ethanol for 1 minute with gentle shaking
    - 70% ethanol for 1 minute with gentle shaking
    - 50% ethanol for 1 minute with gentle shaking
    - 20% ethanol for 1 minute with gentle shaking

  - 0.5% Toluidine blue in 20% ethanol for 30–45 seconds (personal judgment should be used because Toluidine blue improves with age)
- Slides are rinsed with running tap water until the tap water runs clear. (At this point, if the slides are not dark enough when checked under a microscope, dip them in 20% ethanol and put the stain back in; cornified cells should be stained blue with a purple nucleus visible.)
- The stained slides are blotted gently under bibulous paper.
- The wet papers are thrown away because material on a slide can be transferred to other slides.

The slides shall be allowed to dry overnight and then cover-slipped using Permount™ or another suitable mounting medium. If the slides cannot be cover-slipped the following day, store them in a slide folder and coverslip as soon as possible.

9.2.4.1. Slide Labeling and Coding

Each laboratory participating in a study that requires vaginal cytology collection will be assigned an identification number by the sponsor-designated laboratory. (Contact that laboratory to find out your assigned identification number.)

Each slide shall include four lines with the following information (see Figure 9-22 for an example):

(1) C number

(2) Lab code, species, animal number with slide (e.g., 01-M-001A)
  - "01" designates the lab code
  - "M" indicates mouse ("R" would indicate rat)
  - "001A" designates the number randomly assigned to an animal, plus the slide ("A" indicates the first of three slides for that animal, whereas "B" and "C" would indicate the second and third slides, respectively)

(3) The date the first smear was made

(4) The date the last smear was made

C62408 01-M-001A 04-06-06 04-21-06	1	2	3
	4	5	6

C62408 01-M- 001B 04-06-06 04-21-06	7	8	9
	10	11	12
C62408 01-M-001C 04-06-06 04-21-06	13	14	15
	16		

Figure 9-22. Slide Labeling and Coding Example

All decoding information is to remain with the individual testing laboratory and a copy sent to the SMCVC laboratory to be opened only after all evaluations have been completed and in the presence of the Quality Assurance Officer, who will date and sign when opened. Decoding information to be provided is shown in Figure 9-23. All slides will be shipped according to instructions provided in Section 9.2.5.

LABORATORY: XYZ
LAB # 01
TEST ARTICLE: Emodin
ROUTE/VEHICLE: Dosed Feed/NTP2000
CAS # 518-82-1 C # C62408
STRAIN: B6C3F1 Mice
DATES SMEARS TAKEN: 4/6/06–4/21/06
TSAC DATE: 4/22/06

SLIDE CODE	TREATMENT GROUP	ANIMAL NUMBER	BODY WEIGHT @ TSAC (g)
01-M-001	8000 ppm	113	29.2
01-M-002	Control	61	30.6
01-M-003	1000 ppm	85	29.7
01-M-004	2000 ppm	97	28.9
Etc.			

Figure 9-23. Slide Decoding Example

9.2.5. Instructions for Shipping Slides to the SMCVC Laboratory

If required by the study protocol, SMCVC slides shall be shipped to the COR-designated laboratory for evaluation. If slides are to be analyzed by the study laboratory, SMCVC slides shall be submitted to the NTP Archives with the remaining study materials.

- Shipping and packaging shall be incurred by the testing laboratory conducting the toxicity studies.
- Stained and cover-slipped slides shall be randomized by animal and shipped to the sponsor-designated SMCVC laboratory within 2 weeks of necropsy. When randomizing by animal number, keep all slides from each animal together.
- A copy of the code for all slides shall be placed in a sealed envelope and sent to the SMCVC laboratory along with the coded slides.
- All vaginal cytology slides shall be placed in plastic slide boxes, keeping slides from mice and rats separate.
- These slide boxes shall be placed in 350 lb. test cardboard boxes, separated by abundant packaging material for shipment.
- An appropriate listing of slides shall be packed in the slide boxes.
- Slide boxes shall be packed to avoid any breakage.
- Shipping cartons shall be sealed and bound with filament tape prior to shipment.
- Slides sent separately in slide set shall be counted as present in the inventory; a copy of the slide set inventory shall accompany the major inventory document.
- Each plastic shipping box shall be marked with the name and address of the laboratory, the test article, CASRN, NIEHS study number (i.e., C #), strain, and the range of animal numbers included in that box.

- Shipping cartons containing slides and the appropriate information shall be directly mailed to the sponsor-designated SMCVC laboratory.

9.2.6. Suggestions for Improving Vaginal Cytology Quality

General instructions to make vaginal smears are described in detail in Section 9.2.4. A few simple precautions, however, can significantly increase the quality of the slides. Below are suggestions and explanations for correcting potential problems that could be encountered during the preparation of a vaginal cytology smear. A list of vaginal cytology quality codes used by laboratories to evaluate slides is provided in Table 9-1.

Debris. Presence of debris in vaginal smears will interfere during the evaluation. Debris usually comes from dirty slides, tissue fragments, or dust in the air. Therefore, all slides should be precleaned by wiping them with a clean gauze or Kimwipe™. Most of the tissue fragments can be eliminated by carefully aspirating vaginal fluids. It is also important to place all smears that do not have a coverslip in a dust-free environment so dust settling on slides is minimized. Coverslips, if dusty, should be cleaned before use.

Staining. The major problem encountered in this category is nonuniformity of the stain, which can result from the different densities of cells in each smear; however, with optimized automated stainers, smear stain quality is usually sufficient for smear evaluation. With manual stains, one can scan the slide to determine whether all the smears are adequately stained. If the stain quality is poor, place the slides back in the Toluidine blue/Diff-Quik to restain them. Be careful not to stain cells too dark because doing so severely interferes with evaluation.

Clumping and Cell Density. The clumping of cells is mainly due to an excess number of cells on the slide, which can be remedied by not placing too many cells on the slide. If the smear is too dense, it will stain heavily and clump. If too much saline is used in making the smear, the cell density will be very low, not allowing for accurate evaluation. Some stages have inherently high cell density, whereas other stages have very low cell density, thus contributing to this challenge of attaining the ideal number of cells on a slide.

Air Bubbles. Air bubbles are a major problem, especially when they occupy a large portion of the slide. They are mainly caused by faulty technique and/or carelessness. More mounting medium might be needed to cover the entire slide, or better pressing of the coverslip to squeeze out air bubbles could be required.

9.2.6.1. Vaginal Cytology Quality Codes

Table 9-1. Codes Used by the Laboratory for the Evaluation of Slides

Code	Code Description	Subcode[a]	Subcode Description
00	Excellent	-1	Refers to day 1
01	Good	-2	Refers to day 2
02	Moderate crystallization	-3	Refers to day 3
03	Heavy crystallization	-4	Refers to day 4
04	Moderate debris	-5	Refers to day 5
05	Heavy debris	-6	Refers to day 6

Code	Code Description	Subcode[a]	Subcode Description
06	Debris surrounding cells	-7	Refers to day 7
07	Staining too light	-8	Refers to day 8
08	Cornified cells stained lightly	-9	Refers to day 9
09	Staining too dark	-10	Refers to day 10
10	Nonuniform staining	-11	Refers to day 11
11	Cells heavily clumped	-12	Refers to day 12
12	Few cells	-13	Refers to day 13
13	No cells	-14	Refers to day 14
14	Air bubbles (>5% and <20% of the slide)	-15	Refers to day 15
15	Air bubbles (>20% of the slide)	-16	Refers to day 16
16	Cells incorrectly applied to the slide (i.e., applied on the side of the slide lacking the frosted end or not in the appropriate grid on the clear portion of the side of the slide with the frosted end)		

[a]A subcode is used in conjunction with a code when that remark applies to a particular day (e.g., “13-2” means “No cells on day 2”).

9.3. References

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9.4. Peer Review

The Division of Translational Toxicology (DTT) conducted a peer review of chapter 9 within the draft *Specifications for the Conduct of Toxicity Studies by the Division of Translational Toxicology at the National Institute of Environmental Health Sciences* by letter in February 2022 by the expert listed below. Reviewer selection and document review followed established DTT practices. The reviewer was charged to:

1. Peer review the following chapter within the draft Specifications for the Conduct of Toxicity Studies by the Division of Translational Toxicology at the National Institute of Environmental Health Sciences.

- Chapter 9: Fetal Examinations and Vaginal Cytology

2. Comment on the completeness of each chapter.

DTT carefully considered reviewer comments in finalizing this document.

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