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CYTOLOGICAL EVALUATION OF VAGINAL SMEARS IN RODENTS: A NON-INVASIVE APPROACH TO REPRODUCTIVE MONITORING

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ABSTRACT

Vaginal smear cytology is a widely utilized, non-invasive technique for determining the stages of the estrous cycle in laboratory rodents, particularly rats and mice. By examining the types and proportions of vaginal epithelial cells—nucleated epithelial cells, cornified (anucleated) epithelial cells, and leukocytes—researchers can accurately identify the four distinct phases of the cycle: proestrus, estrus, metestrus, and diestrus. This method provides valuable insights into the reproductive status and endocrine function of female rodents, making it an essential tool in various fields such as reproductive biology, pharmacology, toxicology, and behavioral neuroscience. The technique is cost-effective, relatively simple, and allows for repeated monitoring without significantly affecting the animals' physiological state. External factors such as stress, environmental conditions, and animal strain can influence the regularity of the estrous cycle, highlighting the need for controlled experimental conditions. Overall, vaginal cytology is a fundamental tool in laboratory animal research, offering a practical and reliable approach to monitor reproductive physiology and enhance the reproducibility of studies involving female rodents.

KEY WORDS: Reproductive behaviour, Rodents, Vaginal smear.

INTRODUCTION

The word *rodent* originated from the Latin word *rodere*, meaning “to gnaw.” The laboratory rat belongs to the order *Rodentia*. Both rats and mice are in the family *Muridae*, and the term *murine* refers to these animals.[1] Mice and rats are the most frequently used mammalian laboratory animals. Their short generation intervals and high reproductive performance make them ideal models for both basic and applied research.[2] In reproductive function research, especially studies involving female animals, mice and rats are commonly used. This is likely due to their well-characterized estrous cycle and ease of handling.[3] The short and precisely defined length of the estrous cycle in these rodents also makes them highly suitable for such research. Determining the estrous phase is essential for selecting females that will mate successfully when paired with a male, which is critical for achieving timed pregnancy or for tracking estrous as a variable that may influence research outcomes.[4,5]

The estrous cycle can also be used to investigate the effects of drugs and chemicals on reproductive function, which are often reflected as disruptions in the typical morphology, cytology, and histology of reproductive organs, as well as alterations in the duration of specific phases of the estrous cycle.[6] This review provides the basic facts about female rat reproduction and highlights the reproductive characteristics which make the rat an appropriate animal model for research on human reproduction.

ANATOMY OF FEMALE AND MALE REPRODUCTIVE TRACT[7-9]

Genital organs of Mice

The reproductive organs of mice differ significantly between males and females, as well as in their structural complexity compared to humans.

Male genital organs

Testes

It is paired, oval-shaped glands housed in the scrotum, an external pouch situated slightly anterior to the anus. They produce sperm in convoluted seminiferous tubules and testosterone in leydig cells, located in the interstitial tissue. They are responsible for producing sperm (spermatogenesis) and male hormones, primarily testosterone. They are a critical model for studying male fertility due to their physiological similarity to human testes.

Seminiferous tubules

This is the site of sperm production (spermatogenesis). The tubules are lined by two main cell types. The Sertoli cells is of columnar, supportive cells extend from the base of the tubule to the lumen. They provide physical and nutritional support to developing germ cells and are crucial for the blood-testis barrier. The Germ cells tend to various stage of differentiation, populate the space between sertoli cells. They undergo mitosis and meiosis to become mature sperm.

Interstitial tissue

This tissue is located between the seminiferous tubules and contains Leydig cells and other cells. Leydig cells are responsible for producing and secreting testosterone and other androgens. Other cells include the interstitium contains blood vessels, nerves, and immune cells, including macrophages.

Epididymis

It is a tubelike structure attached to the posterior of each testis, divided into the head, body, and tail. It's responsible for sperm maturation and storage before ejaculation.

Extra testicular ducts

The Efferent ducts are a series of 3 to 5 ducts that collect and transport spermatozoa from the rete testis to the epididymis head. Vas deferens are paired tubes that conduct spermatozoa from the epididymis tail to the urethra.

Accessory sex glands

These glands produce fluid that mixes with sperm to form semen. Seminal vesicles are bilateral, sacculated glands located dorsolateral to the urinary bladder. The Prostate glands are divided into four lobes in mice (coagulating gland, dorsal, lateral, and ventral lobes), which surround the urethra. The Ampullary glands are out-pouching's of the vas deferens. Bulbourethral glands also called as Cowper's glands are paired glands located near the base of the penis. The Preputial glands are modified sebaceous glands located in the subcutaneous tissue lateral to the base of the penis.

Penis

It consists of a body and a glans that lies internally within a preputial space. Os penis is a bone within the penis that provides rigidity. Mumps or male urogenital mating protuberance) is a fibrocartilaginous structure that extends beyond the urethral meatus. Erectile tissue includes the corpora cavernosum and corpus cavernosum urethrae.

Female genital organs

Ovaries

It is paired, sphere-shaped gonads contained within a fluid-filled pouch called the ovarian bursa. They produce gametes and hormones. Ovarian bursa is a thin membrane, or bursa, surrounds the ovary and oviductal infundibulum, the funnel-shaped opening of the oviduct. A small, slit-like opening called the foramen of the ovarian bursa (FOB) connects the bursa to the peritoneal cavity. This unique feature is believed to increase the chances of the ovulated egg entering the oviduct.

Oviducts

It is a small, tightly coiled uterine tubes connecting the ovaries to the uterine horns. Fertilization takes place in the ampulla region of the oviduct. Cilia and smooth muscle contractions within the oviduct transport eggs and sperm toward each other for fertilization. After ovulation, a mucus plug temporarily seals the isthmus, keeping oocytes within the ampulla.

Uterus

Mice have a bicornuate uterus with two uterine horns that unite distally to form a short body. This allows for the simultaneous development of multiple embryos, or a litter.

Cervix

It is the narrow opening between the uterus and the vagina. It consists of a single canal that is rich in both circular smooth muscle and collagen fibres.

Vagina

It is a canal that is composed of parts derived from the Mullerian ducts and urogenital sinus. The prominent appendage seen on the outside of the female is actually the prepuce, not the clitoris.

Clitoris

This is an internal organ defined by a u-shaped epithelial lamina and containing a small or clitoris. It does not project externally like the human clitoris and is immobile due to ventral tethering.

Prepuce

It is the external, prominent appendage in female mice is the prepuce, which is bifid and covers the internal clitoris.

Genital organs of Rat
Male reproductive anatomy
Scrotum
The pouch of skin housing the testes is located near the tail and is separate from the abdominal wall, allowing for thermoregulation of the sperm-producing testes. The inguinal canals remain open throughout the life cycle, which means the testes can be retracted into the abdominal cavity during non-breeding periods.

Penis
It is situated in the lower abdomen. The penis is typically housed within a fold of skin called the prepuce. It contains a cartilaginous or bony structure, the *baculum* or *os penis*, which helps with rigidity during copulation.

Anogenital distance
The space between the anus and the genital opening is distinctly longer in males than in females, a key identifier even in newborns.

Preputial glands
A pair of large, modified sebaceous glands located at the base of the penis, which produce secretions involved in territorial marking and communication.

Testes
The male gonads, located inside the scrotum, where sperm is produced. The paired testes are the site of sperm production (*spermatogenesis*) within coiled seminiferous tubules. Interstitial cells, or Leydig cells, produce androgens like testosterone. The testes are enclosed in a protective connective tissue capsule called the tunica albuginea.

Epididymis
A highly coiled tube divided into a head (caput), body (corpus), and tail (cauda) that lies on the surface of the testis. Its primary function is the maturation and storage of sperm before ejaculation.

Vas deferens
This paired duct transports mature sperm from the tail of the epididymis to the urethra during ejaculation. In rats, it joins the urethra near the ampullary glands.

Urethra
A common duct for both urine and semen, which passes through the center of the prostate gland.

Accessory sex glands
These glands produce fluid components of semen. Rats have several, which is a key difference from the human prostate, a single-lobed organ.

Prostate gland: The rat prostate is a multilobed organ, divided into the ventral, dorsolateral, and coagulating glands.

Seminal vesicles: A pair of large, sacculated glands located behind the urinary bladder. They are attached to the coagulating glands.

Coagulating glands: Also known as the anterior prostate, these glands secrete a product that combines with other fluids to form a seminal plug after ejaculation.

Ampullary glands: Outpouchings of the vas deferens that contribute to the seminal fluid.

Bulbourethral glands: Paired glands located at the base of the penis.

Female reproductive anatomy of Rat
Urethral opening and vaginal opening
In females, the urethral opening is separate from the vagina. The vaginal opening is located directly below the urethra.

Anogenital distance
The distance between the anus and the genital openings is significantly shorter in females compared to males.

Nipples
Females develop six pairs of nipples along two "milk lines," visible by 8–14 days of age. Male rats lack nipples.

Clitoris
Located above the urethra and enclosed within a prepuce, it is the female homolog of the male penis.

Preputial glands (clitoral glands)
Exocrine glands near the clitoris that produce pheromones important for sexual attraction.

Ovaries
The female gonads, located at the distal end of the uterine horns, near the kidneys. They produce ova (eggs) and hormones. Each ovary is enclosed by a capsule called the ovarian bursa.

Oviducts
It is a small, coiled tubes connecting the ovaries to the uterine horns.

Uterus
The rats have a bicornuate uterus, with two separate uterine horns.

Cervix
The rat uterus has two separate cervices, with one leading into each uterine horn. The cervices unite where the horns meet the vagina.

Vagina
A muscular canal connecting the uterine horns to the exterior. It serves as the birth canal and for copulation.

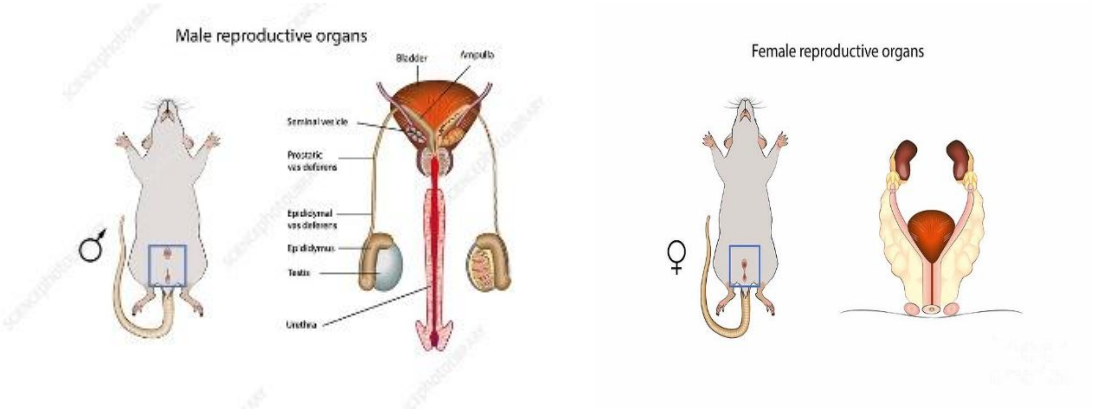

Figure 1: Anatomy of genital tract in rat and mice.

REPRODUCTIVE BIOLOGY AND PRENATAL DEVELOPMENT IN RATS

In rats, puberty typically occurs between 2 to 3 months of age, though this timing can vary from 40 to 72 days depending on factors such as strain or stock. Female rats generally enter puberty earlier than males. In Wistar rats specifically, the first signs of estrus — an early indicator of reproductive maturity — usually appear around 35 to 36 days of age.[10-13]

In Wistar rats, the first signs of estrus — an early indicator of reproductive maturity — typically appear around 35 to 36 days of age. The estrous cycle in rats, which regulates reproductive activity, generally spans 4 to 5 days and continues year-round, remaining unaffected by seasonal changes under laboratory conditions. This cycle comprises four distinct stages: proestrus, estrus, metestrus, and diestrus, each lasting for approximately equal durations within a typical 4-day period. However, occasional variations may lead to extended 6-day cycles, usually due to a prolonged diestrus or proestrus phase.[14]

In prepubertal rats, the uterus may appear as though it contains fluid during the proestrus phase, which can be mistaken for hydrometra and therefore requires careful differentiation. Ovulation — the release of mature oocytes — typically occurs about 8 to 11 hours after the onset of estrus, most commonly between midnight and 2 a.m. However, this timing can vary depending on the light–dark cycle. Once released, the eggs remain viable and capable of fertilization for approximately 10 to 12 hours.[15,16]

The typical gestation period in rats ranges from 21 to 23 days. However, in certain conditions — such as in lactating females — this period may be extended by an additional 8 to 22 days due to factors like blastocyst diapause and delayed implantation. After the 12th day of pregnancy, manual palpation can reliably detect developing foetuses. By day 13, abdominal enlargement becomes noticeable, and mammary gland development is usually evident by day 14. To ensure successful reproduction and reduce the risk of maternal cannibalism, it is essential to provide a calm and stress-free environment for pregnant and postpartum females. The estrous cycle typically resumes within 2 to 4 days following the weaning of pups, signaling the start of a new reproductive cycle.[14,17]

Parturition in rats typically results in a litter size ranging from 6 to 12 pups. The postnatal development of these pups follows a series of well-defined milestones. Their external ears usually open within 2.5 to 3.5 days, and incisor teeth begin to emerge around 6 to 8 days after birth. A full coat of fur generally develops between 7 and 10 days of age. Eye opening occurs around days 14 to 17, and the pups begin consuming solid food by approximately two weeks. In laboratory settings, it is standard practice to wean the pups and transition them to independent living at around 20 to 21 days of age, by which time they have reached a suitable level of physiological maturity.[15,18,19]

VAGINAL CYTOLOGY

The estrous cycle in both rats and mice typically lasts an average of 4 to 5 days. However, occasional 6-day cycles may occur, usually due to the extension of either the estrus or diestrus phase.[20] Compared to rats, mice exhibit less consistency in both cycle length and cycle continuity. For example, a 4-day cycle in mice is more likely to be followed by a 5-day cycle, rather than another 4-day cycle. A variety of factors can influence the duration and regularity of the estrous cycle, including lighting conditions, age, ambient temperature, noise, nutritional status, stress levels, and social interactions.[21-23]

ESTROUS CYCLE

The estrous cycle in rats is composed of four distinct stages: proestrus, estrus, metestrus, and diestrus. Proestrus lasts approximately 12 hours, followed by estrus, which spans about 9 to 15 hours. Metestrus has a duration of around 21 hours, while diestrus — the longest phase — lasts over 57 hours.[24]

Hormones play a central role in regulating the estrous cycle. Gonadotropins, specifically luteinizing hormone (LH) and follicle-stimulating hormone (FSH), are secreted by the anterior pituitary and drive the progression of the cycle. FSH promotes the growth and

maturation of ovarian follicles, while LH triggers ovulation and the formation of the corpus luteum. As follicles develop, levels of estradiol-17β increase, peaking during proestrus. This estrogen peak stimulates the release of gonadotropins, leading to ovulation. The corpus luteum, formed after ovulation, secretes progesterone primarily during metestrus, with levels declining during diestrus. These hormonal fluctuations are closely linked to changes in ovarian structure and vaginal cytology throughout the cycle.[25]

IDENTIFICATON OF VAGINAL SMEAR CYTOLOGY

Vaginal cytology samples are collected over a minimum of 14 consecutive days to assess the stages of the estrous cycle. Although sampling can occur at any time, it is typically performed in the morning after lights-on. Consistent timing each day is essential to reduce variability. Samples may be obtained via vaginal lavage or swabbing.[26]

Vaginal lavage: A sterile plastic pipette was used to gently instill approximately 0.1 mL of sterile physiological saline (0.9% NaCl) into the vaginal canal, with the tip inserted 1–2 mm to avoid cervical irritation. The saline was repeatedly flushed and aspirated until the lavage fluid appeared turbid, indicating adequate cellular recovery. A new sterile pipette tip was used for each rat and discarded immediately after use to prevent cross-contamination.[27]

Swabbing technique: For mice, holding the animal in hand during smear collection is generally preferred. However, for rats, placing them on the cage lid and lifting the tail for sample collection is often more practical than hand restraint. Vaginal smears were collected using a cotton bud moistened with normal saline and were subsequently air-dried.[28]

PREPARATION AND EVALUATION OF SLIDES

Following lavage, a small drop of the sample is evenly spread on a slide to form a thin smear and allowed to air dry. Various metachromatic stains can be used, with Romanowsky-type stains (e.g., Modified Wright's, Wright-Giemsa), Crystal violet and Toluidine Blue O being commonly recommended for vaginal smear cytology due to their effective cell differentiation. Care should be taken to avoid overstaining, and slides must be thoroughly rinsed.[29]

Crystal violet was added drop wise to the smears and kept aside for 3 minutes and the slide were washed in running water (Figure 1C) and observed under light microscope without glycerin (Figure 2). After collection and drying of vaginal smears, direct crystal violet can also be added. But perfect identification of the phase is difficult and hence, addition of methanol is an important step for clear smear observation.[28]

Phases of Estrous cycle[29-39]	Duration		Cells in phases	Fertility status/ Behaviour
	Rat	Mice		
Pro-estrous	10-14 hours	Less than 24 hours	Small, round, nucleated epithelial cells They will be seen in cohesive clusters (grape clusters), sheets, or strands. However, cohesive clusters, sheets, and strands are not always observed, especially in low cellularity samples. No neutrophils will be seen Low numbers of large epithelial cells and keratinized anucleated cells.	Pre ovulatory phase/ Male acceptance at end of phase.
Estrous	24 and 48 hours	12 to 48 hours	Anucleated keratinized epithelial cells Occasional nucleated epithelial cells neutrophils are absent or occasionally observed in late estrus In mice, at the onset of estrus, anucleated cells are smaller and loose clusters or sheets, As estrus advances, the number of these cells typically increases, and they become larger, often arranged in stacks or layers.	Ovulatory, receptive phase/ Male acceptance.
Metestrus	6-8 hours	Lasts up to 24 hours	combination of anucleated keratinized epithelial cells and neutrophils In mice, nucleated cells may appear occasionally throughout metestrus Early metestrus- neutrophils are interspersed with the epithelial cells and tightly packed together or clumped around the cells; It become very high in number Late metestrus- Neutrophil and epithelial cell numbers decrease In mice- polymorphonuclear leukocytes (anucleated	Post-ovulatory phase/ No male acceptance

			epithelial cells), many cornified cells is an early metestrus stage	
Diestrus	48–72 hr	48–72 hr	Decrease in the number of anucleated keratinized epithelial cells. Neutrophil numbers can vary but are usually higher in number Early diestrus- neutrophils may still appear in clumps. Late diestrus- epithelial cells may become more round or be organized in small clumps, indicating proestrus the next day	Quiescent, non-receptive/ No male acceptance.

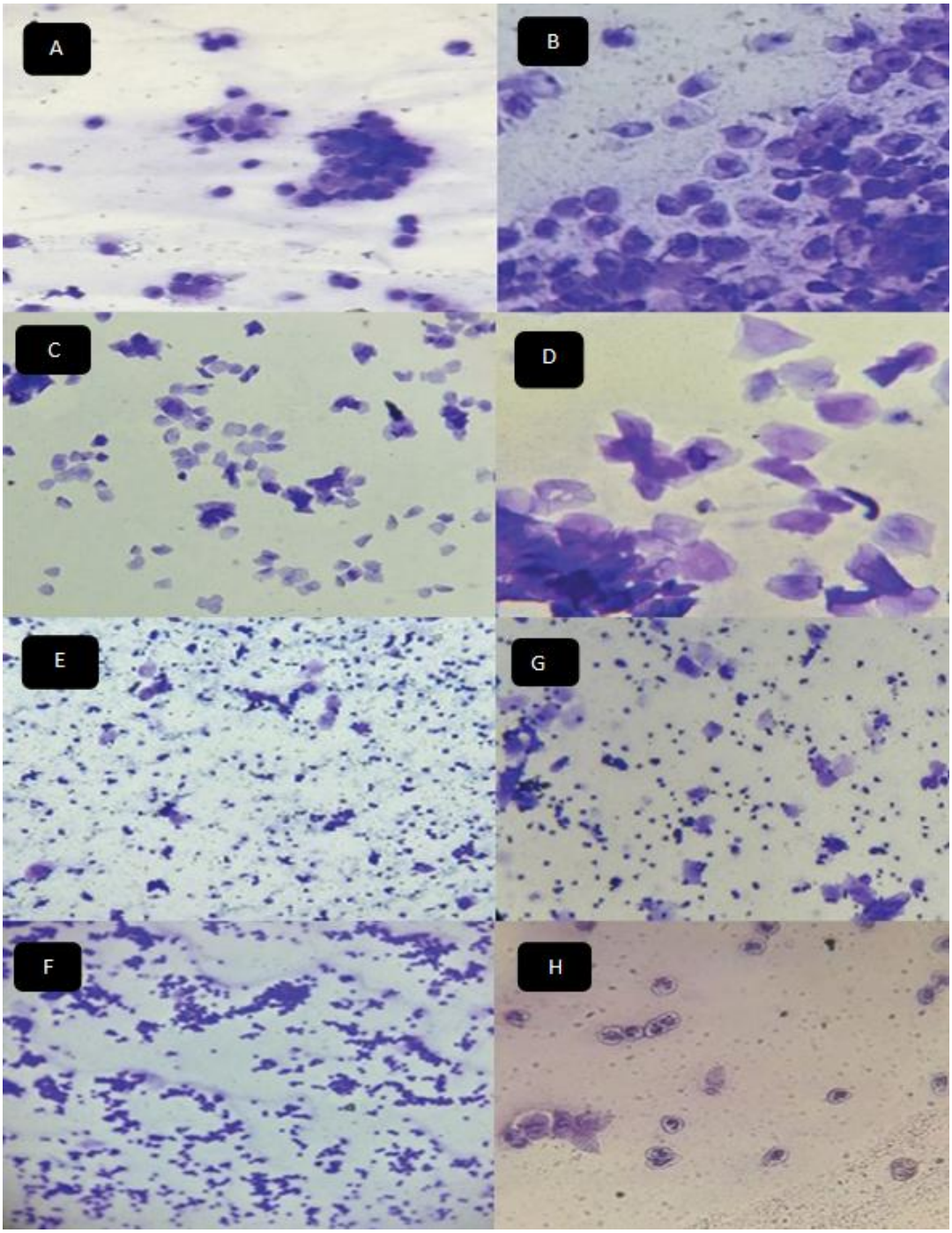

Figure 2: Vaginal smears (Wistar albino rat) stained with crystal violet showed that the various phases of Estrous cycle A & B – Proestrus 10X, 40X respectively, C & D – Estrus 10X, 40X respectively, E & F – Metestrus 10X, 40X respectively, G & H – Diestrus 10X, 40X respectively.

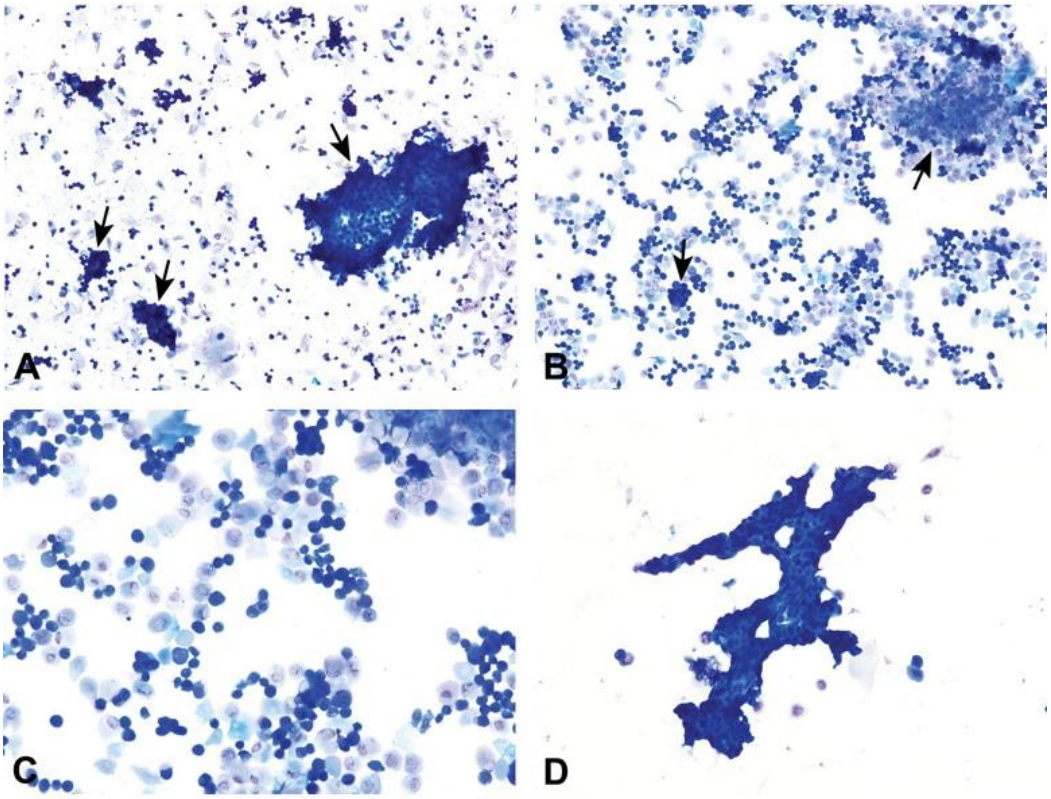

Figure 3: Vaginal smears (Sprague dawley rats) of proestrus stained with Modified Wright–Giemsa stain (A, D) and Toluidine blue O stain (B, C)

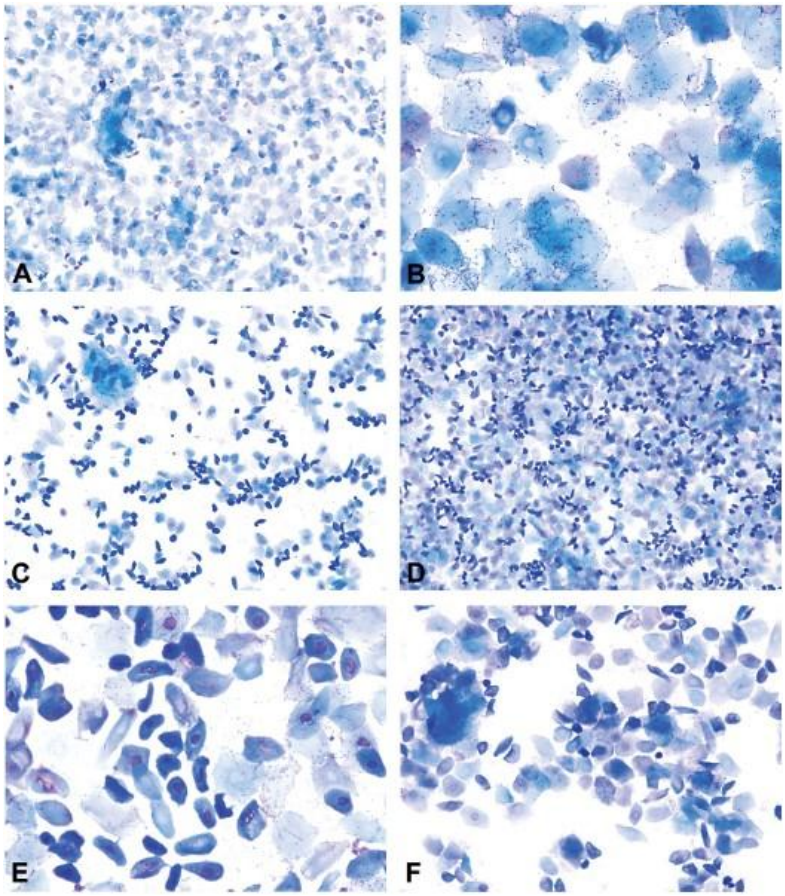

Figure 4: Vaginal smears of estrus (A, B) and late estrus (C–F) in several Sprague-Dawley rats Stained with Modified Wright–Giemsa (A–C, E) and Toluidine blue O (D, F).

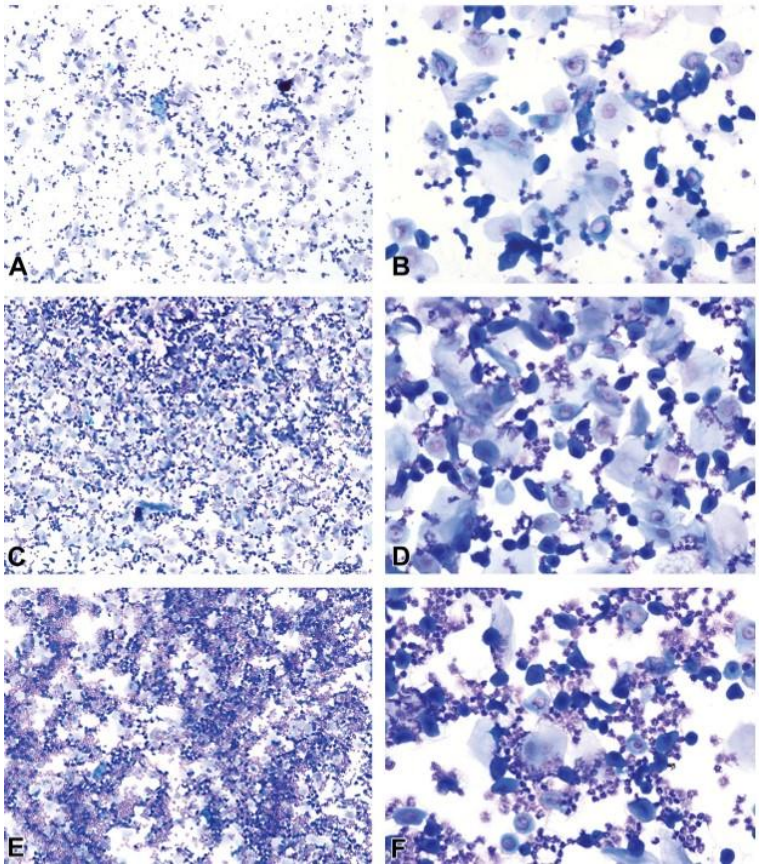

Figure 5: Metestrous vaginal smears from several Sprague-Dawley rats using Toluidine blue O stain. magnification of 10X (A, C, E) or 40X (B, D, F).

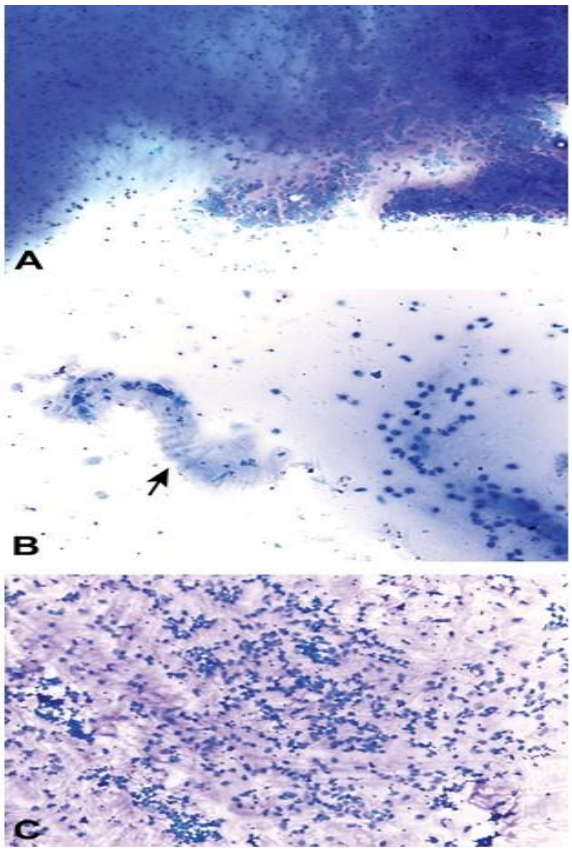

Figure 6: Diestrous vaginal smears from several Sprague-Dawley rats stained with Modified Wright–Giemsa stain (B) and Toluidine blue O stain (A, C).

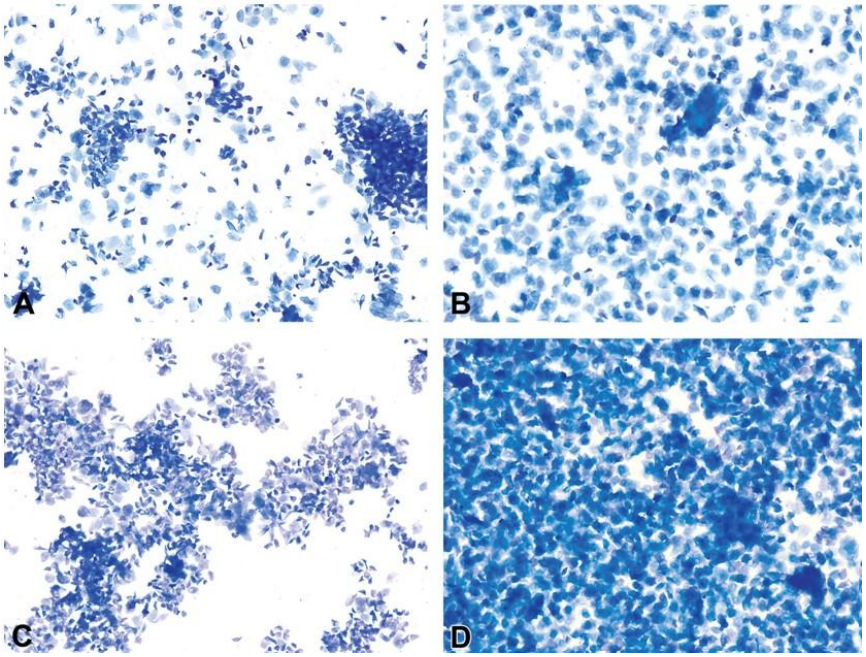

Figure 7: The first (A, C) and second (B, D) "phases" of estrus in mice stained with modified Wright–Giemsa stain (A, B) and Toluidine blue O stain (C, D), magnification is 10X.

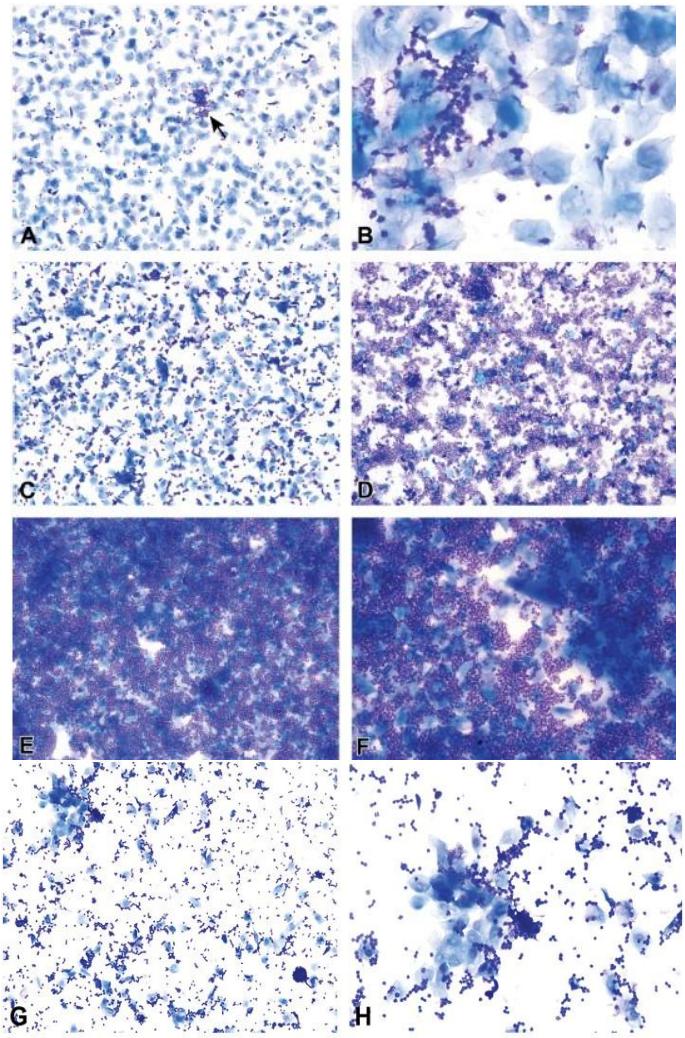

Figure 8: Metestrous smears from mice stained with modified Wright–Giemsa stain (A–D, G, H) and Toluidine blue O stain (E, F), magnification of 10 (A, C–E, G), 20 (F, H), or 40 (B).

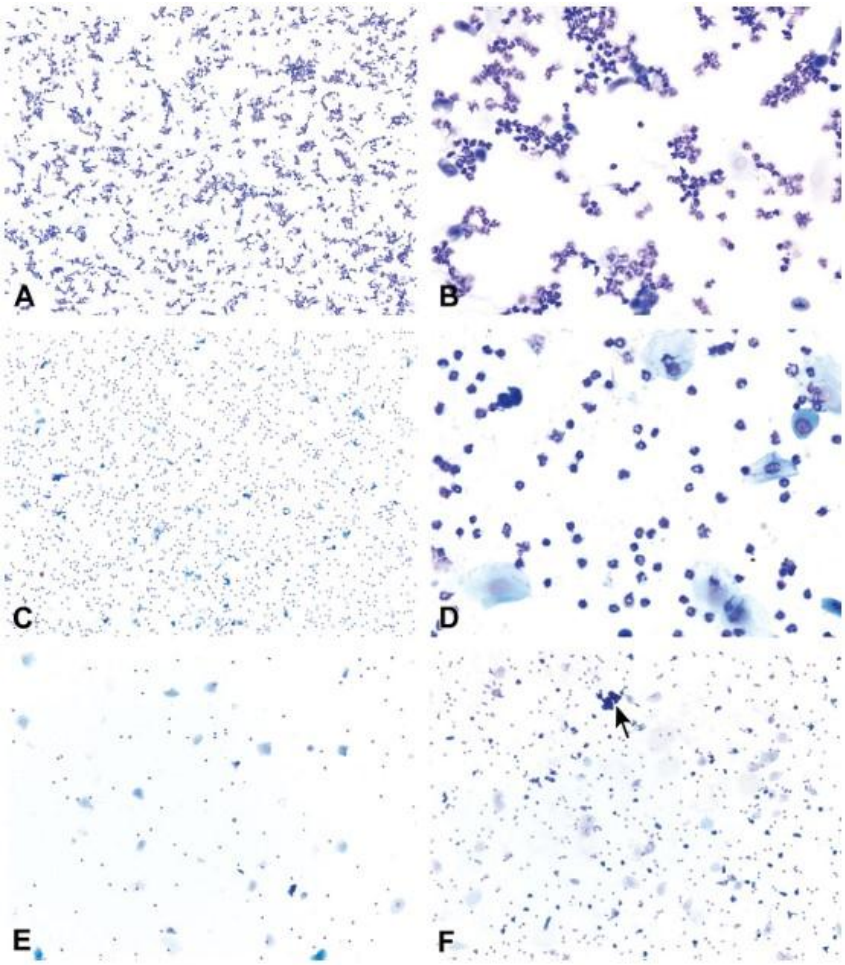

Figure 9: Diestrous vaginal smears from mice stained with modified wright–Giemsa (C–E) and Toluidine blue O stain (A, B, F). magnification of10 (A, C, E, F) or 40 (B, D).

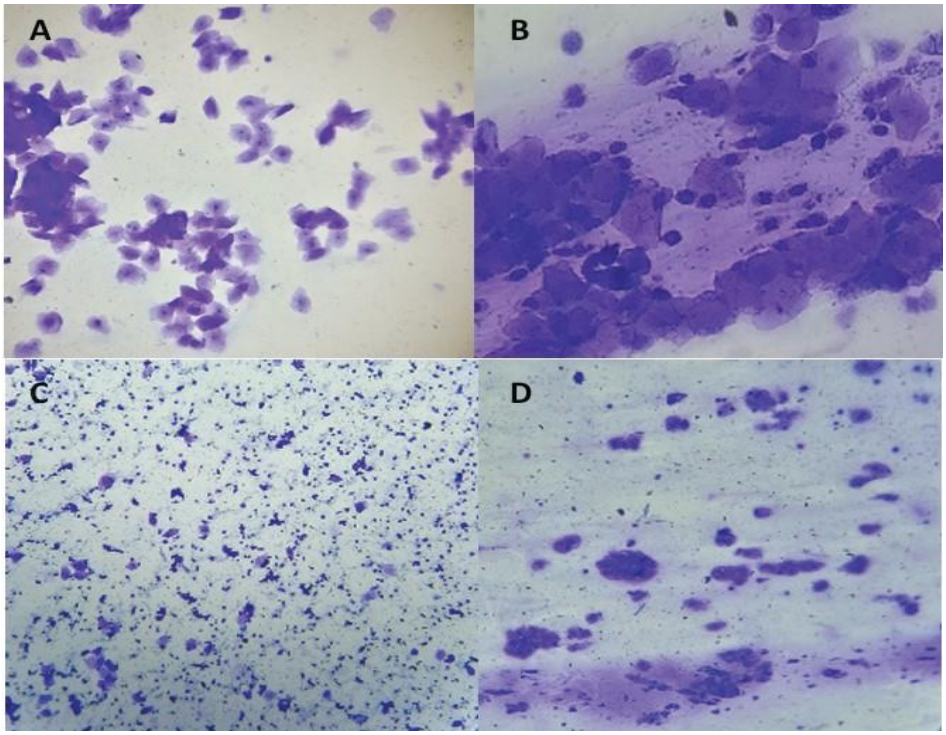

Figure 10: Phase transition between different Phases A: proestrus to estrus, B: estrus to metestrus, C: metestrus to diestrus, D: diestrus to proestrus, 10x Objective lens.

DISCUSSION

Vaginal smear cytology is widely recognized as a cornerstone technique for evaluating the reproductive status and estrous cycle phases in laboratory rodents, particularly rats and mice. This method relies on daily collection and microscopic examination of vaginal epithelial cells, which undergo characteristic changes in response to the animal's hormonal fluctuations. These cyclical cellular changes are directly linked to the underlying ovarian activity and thus provide a window into the reproductive physiology of the animal.

One of the main strengths of this technique lies in its non-invasive nature, making it highly suitable for longitudinal studies where repeated measurements are needed over time. Compared to invasive methods like ovarian histology or serum hormone assays, vaginal cytology is simpler, less stressful for the animal, and does not require specialized equipment beyond basic microscopy. This makes it especially useful in behavioral, pharmacological, and endocrine studies where precise timing of the estrous phase is critical.

Each stage of the estrous cycle presents a distinct cytological profile. For instance, proestrus is dominated by nucleated epithelial cells due to increased estrogen levels, indicating follicular development. Estrus is marked by an abundance of cornified anucleated cells, correlating with ovulation and peak fertility. Metestrus shows a mix of cornified cells and leukocytes, reflecting the early luteal phase, while diestrus is predominantly leukocytic, corresponding to the regression of the corpus luteum and preparation for the next cycle. These patterns not only confirm the animal's reproductive status but also serve as physiological biomarkers for broader studies on fertility, endocrine disruption, and hormonal treatments.

In conclusion, while vaginal smear cytology is a relatively simple and accessible technique, its successful application requires careful handling, consistent methodology, and an understanding of the physiological and environmental factors that influence the estrous cycle. When properly implemented, it remains an invaluable tool in laboratory animal research, particularly for studies involving female reproductive health, endocrinology, and behavioral science.

CONCLUSION

Vaginal smear cytology is a reliable, non-invasive method for determining the stages of the estrous cycle in laboratory animals such as rats and mice. By examining the types and proportions of epithelial cells—namely nucleated epithelial cells, anucleated cornified cells, and leukocytes—researchers can accurately identify the proestrus, estrus, metestrus, and diestrus phases. This technique is essential for reproductive studies, timed mating, hormone research, and understanding physiological responses in female rodents. Its simplicity, cost-effectiveness, and reproducibility make it a valuable tool in laboratory animal science.

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