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# The rat estrous cycle revisited: a quantitative and qualitative analysis

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## Abstract

The rat has been elected as the main animal model in several studies involving reproduction. However, there are scarce and conflicting data related to its estrous cycle. It comprises phases characterized by different cell types in vaginal smears (proestrus, estrus, metestrus and diestrus). Nevertheless, this classification and the time span of each phase are controversial and may induce misleading interpretations. In addition, there are no reports regarding the quantification of all cell types in each phase, including pre-acidophilic cells and leukocytes. The goal of this study was to revisit the literature about the rat estrous cycle and to perform a detailed quantitative and qualitative description of its phases and the transitional periods among them. Vaginal smears were obtained twice daily for 20 days from Wistar rats and stained using the Shorr method. Cells were classified as small (SBC) or large (LBC) basophilic cells, nucleated (NAC) or enucleated (EAC) acidophilic cells, pre-acidophilic cells (PAC) or leukocytes. Ten fields per smear were analyzed and cellular frequencies were determined to distinguish the phases. Enucleated acidophilic cells were observed in all phases. The number of PAC was high during proestrus, but none were found during estrus. Frequency of NAC was higher during the transitional period between metestrus II and diestrus than during other phases. Leukocytes were first observed during metestrus and showed very high frequency during diestrus. This study demonstrated that the quantitative analysis of cell populations in vaginal smears improves the identification of the estrous cycle phases and may contribute to a more precise detection of cyclical alterations.

**Keywords**: estrous cycle, morphometric analysis, rat, Shorr method, vaginal smear.

## Introduction

Female rodents are poliestric, present spontaneous ovulation and show regular and successive estrous cycles that may vary with age and species. These cycles are also influenced by light, seasons of the year and life circumstances. On the other hand, estrous cycles occur without seasonal influence in rats submitted to environmental control under laboratory conditions (Lohmiller and Swing, 2006).

Estrous cycles are characterized by morphological changes in ovaries, the uterus and the vagina (Goldman *et al*., 2007) which occur during different phases called proestrus, estrus, metestrus and diestrus (Hebel and Stromberg, 1986). These phases are usually identified according to cell types observed in vaginal smears. Nevertheless, estrous cycle classifications and the time span of each phase are controversial and can lead to misinterpretations. Female rats' proestrus and estrus phases last for 12 h each, while metestrus lasts for 21 h and diestrus lasts for 57 h. Some authors classify the estrous cycle in five phases (Long and Evans, 1922) as proestrus, estrus, metestrus I, metestrus II and diestrus (Grönroos and Kaupilla, 1959). Metestrus I (or early metestrus) lasts for 15-18 h and metestrus II (or late metestrus) lasts for 6 h (Hebel and Stromberg, 1986). In addition, the cycle has also been divided into proestrus, estrus, diestrus I (or metestrus) and diestrus II (Maeda *et al*., 2000; Westwood, 2008). Maeda *et al*. (2000) also described that in a 4-day cycle diestrus lasts 2 days (diestrus I and II), and in a 5-day cycle, diestrus is extended and lasts 3 days (diestrus I, I and III). Some authors also consider the existence of an additional phase called anestrus which is marked by ovarian inactivity, as observed when reproductive life is quiescent (Westwood, 2008).

Sexual receptivity or "heat" appears only every 4 or 5 days (Westwood, 2008) in the dark period during the estrus phase (Goldman *et al*., 2007; Johnson, 2007). However, females can also accept males during the end of proestrus (Hebel and Stromberg, 1986). The structural changes observed in the vaginal epithelium of female rats during the estrous cycle are induced by estrogen and progesterone. Thus, the rat vagina can be considered a mirror of ovarian function that reflects the activity of sex hormones (Houssay *et al*., 1951).

During proestrus, estrogen level increases and ovarian follicles grow fast (Hebel and Stromberg, 1986; Maeda *et al*., 2000). Ovulation occurs during the night of estrus 10-12 h after the luteinizing hormone (LH) surge. In the absence of mating at the time of ovulation, the corpora lutea are transiently functional and secrete a small amount of progesterone (Johnson, 2007). However, in mated females, 90% of ova are fertilized by sperm cells during the third hour after ovulation and

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luteal life is extended throughout the first half of pregnancy; progesterone production takes place in the placenta during the second half of pregnancy (Hebel and Stromberg, 1986; Maeda *et al*., 2000; Johnson, 2007).

Recently, Marcondes *et al*. (2002) described a fast and simple method to determine estrous cycle phases in unstained vaginal smears, although this method requires more expertise since it is based only on cell shape. Additional information about the stages of the estrous cycle was provided by Hubscher *et al*. (2005), who quantified each cell population found in vaginal smears from rats stained by a modified Papanicolaou method. However, they described only two types of epithelial cells and leukocytes in the smears and did not consider other typical features of vaginal smears such as mucus occurrence. Mucus in smears was referred to by Martins *et al*. (2005) after evaluation of the rat estrous cycle by a liquid-based cytology stained with Evans blue. These authors classified each phase by the observation of three types of epithelial cells (enucleated keratinized cells, intermediate and deep cells) as well as leukocytes; nevertheless, cell quantification was not determined. Therefore, more information regarding the distribution of all cell types found in rat vaginal smears is needed to allow an accurate assessment of the estrous cycle. Thus, the goal of the present study was to achieve a quantification of the cell types during each phase of the rat estrous cycle, as well as during transitional periods between phases.

## Materials and Methods

Ten adult (78 day old, 220 to 240 g) female Wistar rats (*Rattus norvegicus albinus*) were obtained from the Development Center of Experimental Models for Biology and Medicine (CEDEME, Federal University of São Paulo - UNIFESP, Brazil). Five animals were housed per polypropylene cage at the Laboratory of Developmental Biology (UNIFESP, Brazil) under standard conditions of temperature (22 to 24°C), luminosity (12:12 h light/dark cycles) and humidity (60%). Food and water were available *ad libitum*. Experimental procedures were approved by the Institutional Ethics Committee for Experimental Research.

### *Vaginal smears*

To assess the progression of the estrous cycle, vaginal smears were taken twice a day, one in the morning (9 h) and one in the afternoon (17 h), for 20 consecutive days. Considering the short time span of the estrous phases, daily collections were performed with an interval of 8 h to obtain a more precise classification of each phase. Sterile cotton-tipped swabs wetted in distilled water were gently and quickly introduced into the vaginal orifice; the introduction was relatively shallow (approximately 1 cm) to avoid excessive cervical stimulation and a consequent pseudo-pregnancy (Goldman *et al*., 2007). Subsequently, they were carefully rotated (one twist) against the vaginal wall. Rats were not anesthetized during smear collection.

Afterwards, the collected sample of vaginal epithelial cells was placed on glass slides, dried at 37°C and fixed in an ethanol-ether solution (1:1) for one minute. The smear was stained according to the Shorr method (Shorr, 1941), which improves the identification of different epithelial cells. With this method, the cytoplasm of epithelial cells stains a dark orange (acidophilic cells) or blue (basophilic cells), depending on the degree of cell keratinization. Vaginal smears were immersed in 70% ethanol for 1 min, hydrated in water, stained with Harris hematoxylin for 20 sec and quickly washed in distillated water. After this, the smears were dehydrated in 70 and 90% ethanol for 1 min and stained with Shorr stain (Bierbrich scarlet, orange G and fast green - Dinâmica®) for 30 sec. Then, the smears were again dehydrated in 90 and 100% ethanol followed by diaphanization in xylene, mounted under coverslips with Entellan and observed under an Olympus photomicroscope.

### *Morphometric analysis*

Stained vaginal smears were observed under a light microscope and the number of epithelial cell types in each phase of the estrous cycle was determined. One estrous cycle was defined as the number of days from one estrus to the next estrus. Vaginal smears were classified by morphology and staining properties of epithelial cell types desquamated from the vaginal epithelium, as well as the presence or absence of leukocytes and mucus. The morphometric analysis was performed as follows:

a) Morphology and staining properties of epithelial cells*:* epithelial cells were classified as small (SBC) or large basophilic cells (LBC), nucleated (NAC) or enucleated acidophilic cells (EAC) or pre-acidophilic cells (PAC, cells exhibiting a mix of blue and orange staining);
b) Frequency of cell types: the frequency (F) of each cell type (classified according to morphological analysis and staining properties) was determined and expressed as a percentage. The scores of cell types per rat were obtained in each slide independently of the immediate identification of the phase evaluated.

The aim of this procedure was to obtain the complete profile of the estrous cycle for each animal and then, from these observations, to characterize the transitional phases of the estrous cycle. For these analyses, epithelial cell types were counted (N) using a square eyepiece coupled to a light microscope. One hundred cells were randomly scrutinized per slide at 400X magnification. At least 10 slides from each phase

were scored. Thus, each epithelial cell type frequency was calculated by mean number of cells divided by total number of slides in the specific phase. Cell frequency was expressed as a percentage.

c) Number of leukocytes*:* the average number of leukocytes was estimated on a scale of 1+ to 4+ as described: + (up to 10/field); ++ (11 to 25/field); +++ (26 to 50/field); ++++ (more than 50/field);
d) Diameter of cell types: the diameters of different cell types were estimated by measuring their major axis using a Leica QWin V3 image analysis system (Leica, Cambridge, England). Ten cells of each type were measured per slide in each determined phase.

*Statistical analysis*

Statistical analysis was performed using one way analysis of variance (ANOVA). The multiple comparison test (Student-Newman-Keuls) was also used when the differences were statistically significant ($P \leq 0.05$).

## Results

After collecting vaginal smears twice a day from each animal for 20 consecutive days, we did not observe any change in the length of their estrous cycles. Female rats presented normal cycles with a mean duration of 4.5 days.

The majority of smears showed typical features of the known phases of the estrous cycle and could be easily identified. However, the identification was not simple in smears with transitional characteristics. In these smears, cellular features from previous and subsequent collections were evaluated to enhance accuracy in phase determination. The proportion of each cell type varied considerably in these vaginal smears. Different epithelial cell types were identified in the stained smears as depicted (Fig. 1A-J).

Leukocytes (Lkc) were observed in some phases and displayed a central nucleus with several lobules that allowed their identification as neutrophils (Fig. 1C). Basophilic cells were stained in blue and represented less differentiated cells from basal and intermediate epithelial layers. Cells from the basal layer were smaller than the intermediate layer cells and were classified as small basophilic cells (SBC). These cells measured 25 to 32 μm in diameter and exhibited a rounded shape with large nuclei and sparse chromatin. Cells from the intermediate layer were classified as large basophilic cells (LBC). These cells measured 36 to 40 μm in diameter and were polygonal; their nuclei were smaller when compared to small basophilic cells and displayed condensed chromatin (Fig. 1H). Acidophilic cells from superficial layers were more differentiated and stained in dark orange due to the accumulation of keratin filaments. Nucleated acidophilic cells (NAC) were characterized by a polygonal shape, measured 22 to 38 μm in diameter and displayed small and pyknotic nuclei. As the keratinized cells moved to more superficial layers they lost their nuclei. These cells were named enucleated acidophilic cells (EAC). These cells were flat with a somewhat irregular outline and their major axes ranged from 40 to 52 μm (Fig. 1J). In this study, the partly keratinized superficial cells showing a heterogeneously stained cytoplasm (blue and dark orange) were also scored. These cells, named pre-acidophilic cells (PAC), measured 30 to 44 μm in diameter and were polygonal with small nuclei (Fig. 1I).

Stained smears obtained from the vaginal epithelium allowed the characterization of each stage, including transitional periods, during the estrous cycle (Table 1). As previously explained, the term "transitional periods" was used to describe smears collected between two sequential phases that did not reflect typical features.

Scores of epithelial cell populations and leukocytes in each stage of the estrous cycle are shown (Fig. 2). Enucleated acidophilic cells were observed in all phases but were more abundant during the transition from proestrus to estrus and during the estrus stage. At estrus, the number of these cells was much higher than any other cell type ($P < 0.001$), comprising 90% of total epithelial cells. After estrus, EAC number declined and started to increase again during the diestrus phase. These cells were seen aggregated in clumps during estrus and metestrus I.

The population of NAC was higher during proestrus when compared to other phases. During this phase, significant differences among the frequencies of these cells and LBC and PAC were not observed. However, NAC population was greater ($P < 0.001$) than SBC. Pre-acidophilic cells started to increase at the end of diestrus and were at their maximal number during proestrus (11%), similar to LBC frequency. Few SBC were seen during the cycle; these cells were more frequent during the transition from diestrus to proestrus (3%).

The absolute number of leukocytes was not obtained because they occurred in very large quantities during some phases. A relative score (<10; 11 to 25, 26 to 50 and >50/field) was performed, instead. The frequency of leukocytes increased progressively during the transition from estrus/metestrus (Fig. 1B) to diestrus (Fig. 1F, 1Ga and 1Gb). Leukocyte frequencies throughout the cycle are shown (Fig. 2). The number of these cells declined during the transition from diestrus to proestrus and they were never seen during the proestrus stage.

Thick mucus was noted during the diestrus phase. Our observations showed that the mucus appeared during the transition from metestrus II to diestrus, becoming more abundant at mid-diestrus (Fig. 1Gb).

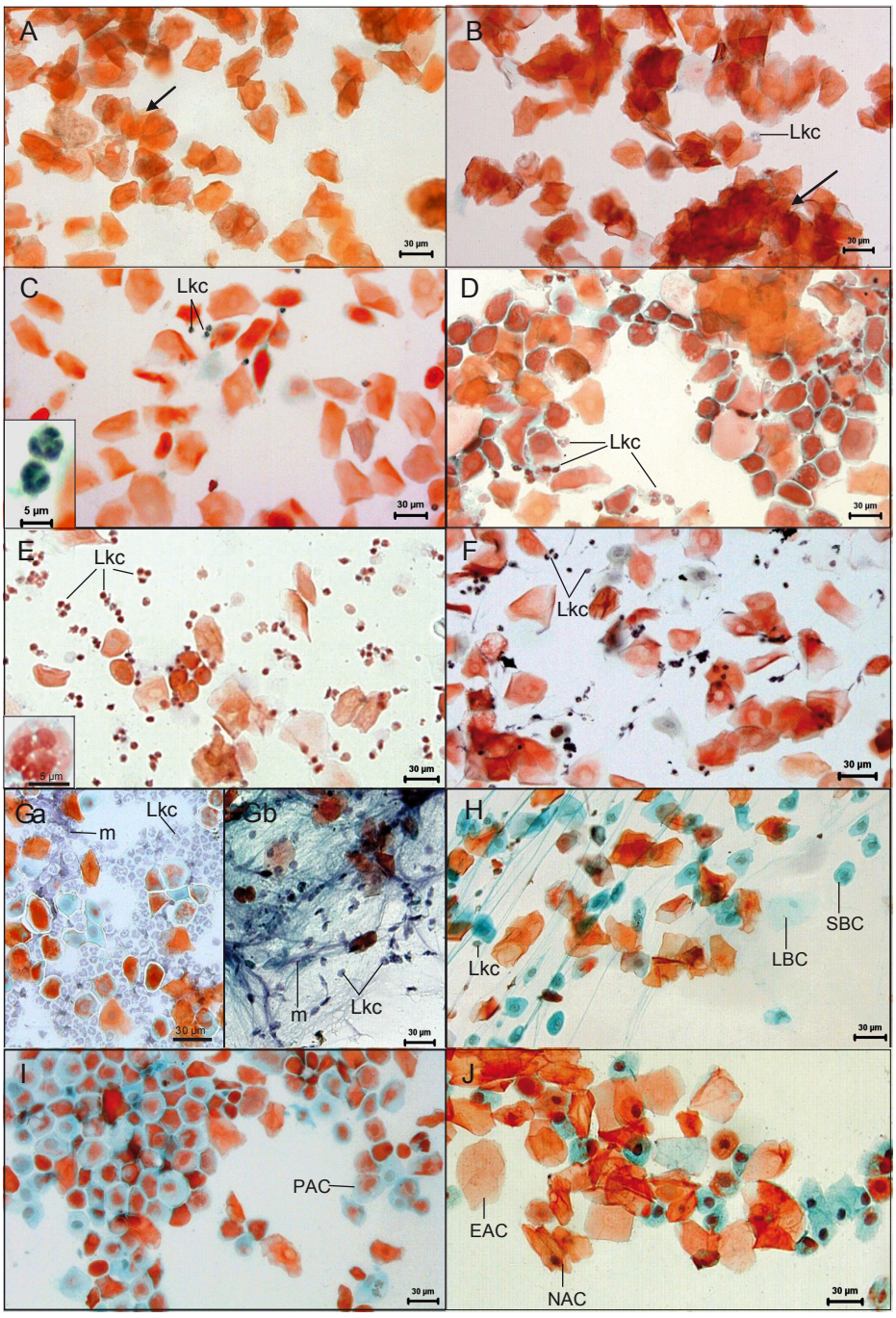


Figure 1. Vaginal smears stained by the Shorr method during each phase and during transitional periods of the estrous cycle. **A:** During estrus, clumps (arrow) of enucleated acidophilic cells are observed. **B:** Transition from estrus to metestrus I; clumps (arrow) and leukocytes can be seen. **C:** During metestrus I, leukocytes are also observed and shown in the insert. **D:** Transition from metestrus I to metestrus II. **E:** Metestrus II, showing a high number of leukocytes; also shown in the insert. **F:** Transition from metestrus II to diestrus. **G:** Mucus (m) stained in blue, numerous leukocytes can be seen during early diestrus (**a**); leukocytes appear trapped within abundant mucus during mid-diestrus (**b**). **H:** Transition from diestrus to proestrus. Observe a small basophilic cell (SBC) and a large basophilic cell (LBC). **I:** Proestrus, with pre-acidophilic cells (PAC). **J:** Transition from proestrus to estrus, when nucleated (NAC) and enucleated (EAC) acidophilic cells can be observed.
Leukocytes are represented by "Lkc" in the images.

Table 1. Main features during phases and transitional periods of rat estrous cycles.

| Phases | Features | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | SBC | LBC | PAC | NAC | EAC | Clumps | Lkc | Mucus |
| Estrus | - | - | - / + | ++ | ++++ | ++ | - | - |
| Estrus / Metestrus | - | - / + | - / + | ++ | +++ | +++ | + | - |
| Metestrus I | - | - / + | - / + | ++ | +++ | +++ | + / ++ | - |
| Metestrus I / Metestrus II | - | + | + | + | ++ | - / + | ++ / +++ | - |
| Metestrus II | - | + | + | + | ++ | - | +++ | - |
| Metestrus II / Diestrus | - / + | + | + | + | ++ | - | ++++ | - / + |
| Diestrus | + | ++ | ++ | ++ | ++ | - | ++++ | + / ++ |
| Diestrus / Proestrus | + / ++ | ++ | ++ | ++ | +++ | - | + | - / + |
| Proestrus | + / ++ | ++ | ++ | +++ | ++++ | - | - | - |
| Proestrus / Estrus | + / - | + | + | ++ | ++++ | - / + | - | - |

Small basophilic cells (SBC), Large basophilic cells (LBC), Pre-acidophilic cells (PAC), Nucleated acidophilic cells (NAC), Enucleated acidophilic cells (EAC), Grouped EACs (Clumps), Leukocytes (Lkc). Absent (-), low (+), moderate (++), high (+++), very high (++++).

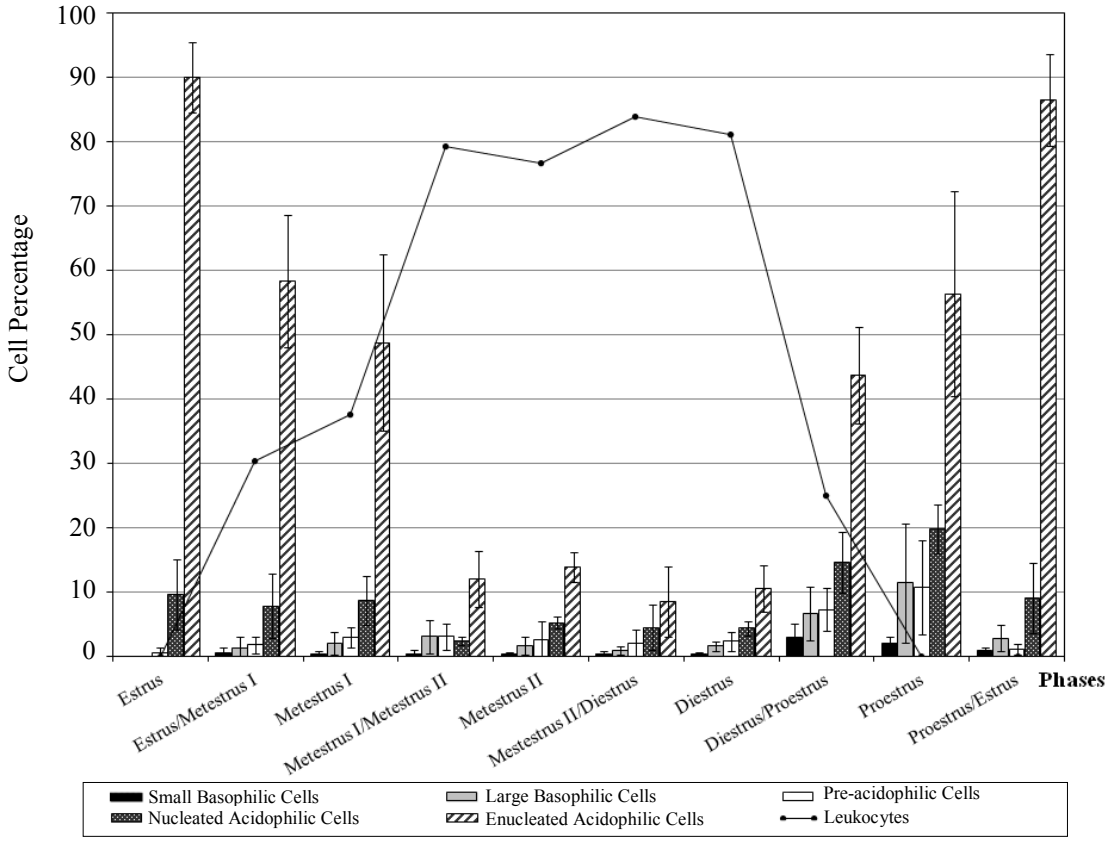


Figure 2. Scores of epithelial cell populations and leukocytes during each phase of the rat estrous cycle and transitional periods between them.

## Discussion

In this research, the colpocytological method was used to achieve a detailed morphological and quantitative description of each phase of the estrous cycle; transitional periods between the subsequent main phases of the cycle were also considered. Vaginal smears were obtained using sterile cotton-tipped swabs wet with distilled water, a fast and suitable method of collection. This method seems to be less aggressive than the micropipette technique (Yener *et al*., 2007). In this experiment, distilled water was preferred since Andersen *et al*. (2004) showed that saline can be crystallized on slides after liquid evaporation and can hinder the identification of the phases, especially diestrus.

Previous studies suggest that frequent stimulation caused by successive vaginal epithelial cell collections can induce the prolongation of the luteal phase, indicating pseudo-pregnancy (Johnson, 2007). Besides, near ovulation, alterations in the frequency, intensity and duration of the cervical stimulation caused by the collection can mimic the coitus (Gunnet and Freeman, 1983; Yener *et al*., 2007). This stimulation is relayed via sensory nerves from the cervix to the central nervous system and activates prolactin release from the pituitary gland (Johnson, 2007). In rodents, prolactin maintains the luteal phase and, consequently, progesterone secretion up to 12 to 13 days, delaying the next estrous cycle (Gunnet and Freeman, 1983). In the present study, the collection was performed during 20 consecutive days (twice a day) from each animal and the induction of pseudo-pregnancy was avoided by gentle smear collection. The frequent stimulation did not cause changes in estrous cycle length.

Due to the influence of the endogenous circadian system on ovulation (Schwartz, 1982) and on the length of estrous cycle phases, mainly during estrus, animals were maintained under rigid control of light (12:12 h light/dark cycles). In fact, the secretion of gonadotropin-releasing hormone (GnRH) and luteinizing hormone (LH) in a given moment of the day is regulated by a neuroendocrine integrative mechanism that depends on the levels of estradiol produced by the ovaries and on circadian signals (de La Iglesia and Schwartz, 2006). In the present study, it was observed that the average duration of estrous cycle was 4.5 days, ranging from 3.5 to 5.5 days (data not shown). This observation agrees with those described by Mandl (1951) and Bertalanffy and Lau (1963). On the other hand, Grönroos and Kaupilla (1959) showed that the average length of the rat estrous cycle was 4.8 days, ranging from 3 to 8 days. The duration of each phase of the estrous cycle was: proestrus and estrus 12 h each; metestrus I, 15 to 18 h; metestrus II, 6 h; and diestrus, 57 to 60 h. These results are in accordance with the findings of Long and Evans (1922), who divided the estrous cycle into 5 stages. In addition, it is important to consider that abrupt changes in the distribution of typical cell types are not observed from one phase to the next in vaginal smears (Bertalanffy and Lau, 1963), indicating that transitional phases occur.

Thus, the transitional periods were identified in rat vaginal smears by exhibited features which are common to consecutive phases (e. g., leukocytes, often during diestrus, associated with numerous small basophilic cells, found during proestrus). The determination of transitional periods between the specific stages was helpful to clarify those periods without typical hallmarks, which lead to misinterpretation. This can be illustrated by the predominance of superficial enucleated acidophilic cells during all the cycle, although their frequency was lower during the transitional period from metestrus II to diestrus. The nuclear and cellular features were also carefully observed to differentiate the phases. Thus, the degeneration of epithelial cells which occurred at the end of metestrus II and the increase of their frequency during the transitional phase from diestrus to proestrus were useful in identifying such stages.

In contrast to the present study, other researchers (Long and Evans, 1922; Marcondes *et al*., 2002; Hubscher *et al*., 2005) described epithelial cells with only two or three morphological types. On the other hand, in the present study, five epithelial cell types (nucleated and enucleated acidophilic cells, small and large basophilic cells and pre-acidophilic cells) were observed. It is important to emphasize that this classification was based on cellular measurements and staining properties of epithelial cells found in smears. These cells were stained in blue or dark orange by the Shorr method and represented different epithelial layers of the rat vaginal epithelium. Our observations showed differences in the types of epithelial cells in rat vaginal smears when compared to the findings of Hartman (1944). Although this author also used Shorr method, a different classification was used to describe the cells, which included mucous cells and phagocytized cells besides six subclasses of epithelial cells. Grönroos and Kaupilla (1959) also quantified the epithelial cells from different layers of vaginal epithelium and classified them as superficial, intermediate, basal and parabasal cells in both normal and stressed rats. Nevertheless, statistical analysis was not performed.

Pre-acidophilic cells were maximal during proestrus, making the identification of this phase very easy in stained smears. Cytoplasms of epithelial cells heterogeneously stained in blue and orange by the Papanicolaou method were previously observed in rat smears by Hubscher *et al.* (2005). However, these authors only quantified nucleated and enucleated cells without considering the different staining patterns.

The same authors reported that leukocytes were found in all stages of the estrous cycle (Hubscher *et al*., 2005). Nevertheless, we did not observe these cells during the proestrus and estrus stages. These results are similar to those obtained by various authors (Long and Evans, 1922; Grönroos and Kaupilla, 1959; Centola, 1978; Montes and Luque, 1988) who noticed leukocytes only during the metestrus and diestrus phases. The presence of leukocytes has also been associated with high progesterone levels, which induce an influx of neutrophils into the vagina (Johnson, 2007). Another point to be considered is the fact that metestrus I vaginal smears exhibited clumped enucleated acidophilic cells, and this characteristic is similar to those observed during the estrus phase. Thus, fewer leukocytes were found in smears during metestrus I, enabling the distinction between estrus and metestrus. A sequential cytological analysis of the cycle, as well as the careful analysis of vaginal smears, was essential to the identification of these phases. Besides, two collections with an interval of 8 hours,

were also useful to avoid doubts about possible changes during the estrous cycle.

After dried, female rat fresh smears exhibited mucus in a fern leaf pattern similar to that observed in humans (Owen, 1975; Menárguez *et al*., 2003). In rodents, the presence of mucus in diestrus smears can be considered a key characteristic in distinguishing estrous cycle phases (Grönroos and Kaupilla, 1959; Bertalanffy and Lau, 1963; Centola, 1978; Martins *et al*., 2005). The occurrence of mucus stained in blue by the Shorr method, associated with the presence of leukocytes, was helpful to facilitate the differentiation between the metestrus II and diestrus phases. The present study showed a quantitative and qualitative analysis of the rat estrous cycle, including a description of the transitional periods and a detailed description of the epithelial cell types present in the smears. In addition, a statistical analysis was performed to confirm the relevance of the differences among the estrous cycle phases. A detailed classification of the estrous cycle phases is essential for studies using pre-implantation and just implanted embryos, for example, since it facilitates experiment programming. Finally, this study provided a revision of the different strategies used to analyze the estrous cycle described in the literature combined with the procedures performed here, which can contribute to researchers whose studies rely on detailed information about the rat estrous cycle.

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