Liquid-base cytology: a new method for oestral cycle study in wistar's rats[1]

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Martins RR, Pereira NML, Silva TMA. Liquid-base cytology: a new method for oestral cycle study in wistar's rats. Acta Cir Bras [serial on line] Available from: URL: htt://www.scielo.br/acb.

ABSTRACT – Objectives: The objective of this study was the standardization of a collection technique and staining in liquid-base that allies the pratical and cytological wealth, making possible a larger reproductibility and microscopic easiness. **Methods:** Female wistar rats (n=20) were submitted to the daily vaginal collection in saline and fastened washed (ether/alcohol) and stained in suspension with a solution of Evans Blue 0.025%. The sample was pondered by centrifugation and observed under lens of 40 x. **Results:** The stained smears allowed clear differentiation of the phases of hormonal cycle (diestrus, proestrus, estrus and metestrus); besides the differentiation of the cellular types in relation to its maturation degree having as parameters the cellular size, nucleus / cytoplasm relationship (NCR) and ink reaction. The study demonstrated the existence of three basic cellular patterns: cells with low NCR, accentuated cyanophily and small size; cells with increment in NCR, cyanophilic loss and larger volume cytoplasmatic and without nuclei keratinization cells in squamous aspect. **Conclusion:** The staining of the material allowed, besides the cytological classification, the quantification possibility that would result in a perfected accompaniment of the cycle estrous.

KEYWORDS: Vaginal epithelium, cycle estrous, base-liquid cytology, Evans Blue.

Introduction

The study of the oestral cycle in wistar rats is an important experimental source due to its short duration and easy observation of cytological characteristics. Simple techniques based in the daily fresh collection represent an important tool in the study of reproductive and pharmacological aspects[1, 2].

Since the first works of Stockard and Papanicolaou (1917), Long and Evans (1921) and Allen (1922) it had been well demonstrated that oestral cycle can be described through changes in the observed cellular standards in the collection of vaginal flow[3].

Through fresh cytology, its evidenced the levels of the reproductive cycle, which begins with abundant keratinization of the epithelium and absence of leukocytes, this period is named estrus; physiologically it corresponds to the maximum estrogenic stimulation within the tissue. After that the keratinization diminishes gradually allowing the appearance of nuclei cells and leukocytes, this period is named metestrus. The diestrus presents predominance of leukocyte infiltration and nuclei cells. The proestrus period presents reduction of leukocytes, increase of the nuclei cells number and presence of keratinization. The total duration is established around four days, but cycles with five days also can be seen[3, 4, 5, 6, 7].

The maturation of the vaginal epithelium is determined by the local action of the steroids hormones that promote changes in cell size, nucleus-cytoplasmic relation (NCR) and reaction to basophilic and acidophilic dye[8].

The epithelium near the basal lamina shows rounded shape cells with an increasing relation nucleus-cytoplasm and marked cyanophily, as the tissue gets mature, it initiates a process of increase of the cellular volume and nuclear involution culminating with the loss of nucleus[3].

Literature presents some studies on the systematization of the oestral cycle related to total duration and its characterization in phases. However, the execution of the analysis of the samples can present difficulties to the microscopist not used to the murine cytology.

Our study aims to validate a technique of collection and coloration in liquid-base that allows better conditions in the characterization of the oestral cycle; with a adjusted collection procedure that unites practical manipulation with microscopical abundant

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number of cells. Besides correlating the phases with mature aspects of the epithelium and other cytological characteristics.

Methods

Animals

It had been used young females (*Rattus norvegicus albinus*, mammalia Rodentia) aging between 90-100 days and weighting of 180-220g, separated in groups of five for cage kept under

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controlled temperature and illumination (24-27°C and daily illumination between 7:00 and 18:00h), with standardized ration and water *ad libitum*. The experiment was lead in the installations of the Central Bioterium of the Center of Health Sciences of the Federal University of Rio Grande do Norte, Natal-Brazil. The animals had been submitted to the daily vaginal collection per two weeks before the beginning of the study, for individual following up of the cycle. Only specimens with regular cycles had been used. In this work the constant recommendations of the International Guide of Principles for Biomedical Research Involving Animals had been followed.

Collection and Coloration

During 30 days, it had been accomplished a morning collection between 8 and 9h in diverse enclosure of the captivity environment. Pasteur pipettes with 151 mm of length and diameter of 2 mm had been used. The vaginal secretion was collected through the introduction of 250 mL of saline solution (NaCl 0.9%) inside the vaginal cavity. The content was mixed in 1 mL of fixing solution (ether/alcohol absolute 1/4) and left in rest per 30 minutes.

The material was centrifuged at 700g per 5 minutes and the supernatant was thrown away. The sediment was restart in 1 mL of Blue Evans (BE) 0.025%, remained in rest per 20 minutes and the centrifuge procedure was repeated. Then 25 mL of the sediment was observed between slide and lamella in the objective of 10 and 40x. 600 samples had been evaluated during our study.

Determination of the cycle period and its duration was done considering the cellular composition of the smear and the relative ratio between the peel cell types; leukocyte

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infiltration, presence of mucus and the appearance of the centrifuged sediment. In case of difficulty in the identification of proestrus, a retrospective analysis and the occurrence of following estrus confirms or not its presence.

Results

In the smears, it has been observed three types of peeling cells: anuclei keratinizated cells, intermediate and deep cells.

The anucleai cells are polygon and of bigger volume, presenting raised cytoplasmic transparency. It did not have affinity to the BE (figure 1a). The intermediate cells had presented a smaller cell size and centered nucleus of bigger volume (figure 1b). The deep cells are smaller, spherical and lacking cytoplasm (figure 1c).

The intermediate cytoplasm was stained in blue having bigger nuclear definition; while that in deep the cyanophily was intense, but still allowing the delimitation of the nucleus (figure 1d), its observed a correlation between the cellular maturation and a lesser affinity for the dye. The leukocytes stained moderately when they were present.

The diestral phase is classically characterized for cellular lacking and leukocyte infiltration, however, the procedure allowed the visualization of satisfactory deep cells in amount (figure 2a). In proestrus, it takes place an increase of the mature cells aspects with an increasing amount of intermediate and superficial cells; the stain selectivity of the BE enhanced the ratio between the present cellular types (figure 2b). The superficial cells reach the totality of smear in estrus; the collected material showed cytological abundance and little chromo affinity with significant cellular overlapping (2c). Metestrus is initiated with the appearance of leukocytes [5]. Smear becomes scarce and presents mature elements, nuclei cells and deeply stained, still presents keratinization (2d).

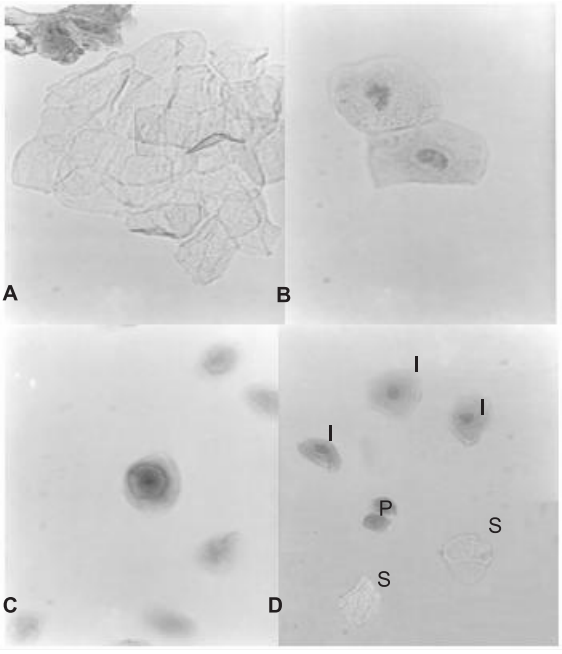

FIGURE 1– (A) superficial Cells presenting low chromo affinity for the blue Evans (BE), it is observed nucleus absence and the polygon format (40 x), (b) intermediate cells (i) moderately cyanophily with not common nucleus and dense chromatin (100 x), (c) deep cell (p) with spherical format and lesser cellular size, nucleus shows intensely stained for the BE, (d) staining affinity decreases with the degree of cellular maturation

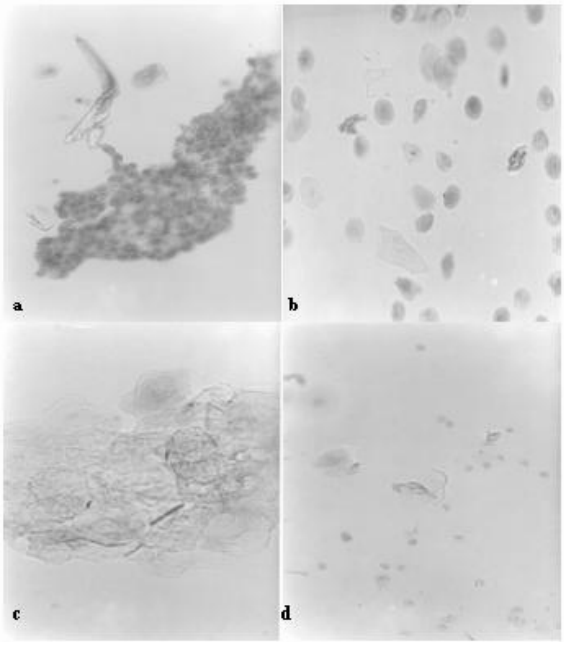

FIGURE 2 – (a) diestral Phase presenting accented amount of cellular material (10 x), (b) Proestrus, is distinguished the diverse selectivity of the staining for each cellular type (40 x), (c) characteristic cellular overlapping of estrus, observes it low chromo affinity in this phase (40 x), (d) metestrus.

The whole time needed to collect and sample process in the 20 studied specimens, was around one hour and a half. The average duration of the estral cycle of the evaluated group was of 4 +/- 0,5 days.

Discussion

The collection procedure revealed to be little aggressive and free of stressing factors beyond the necessary for the animals. The cellular material presented conservation and adequate cytological abundance for the study, despite the diestral period being scarce in peeling, the centrifuge made possible the adequate analysis. Solution of ether/alcohol ¼ showed to be efficient in the cellular conservation in suspension, making possible the study for a superior period the 24 hours the collection. The blue Evans (CI 23860) is a basic staining that promotes cyanophily to the nuclear structures of cells 10. The synthesis and accumulation of keratin that occurs in the cellular maturation reach its maximum with the presence of superficial anuclei cells [1]. These cells possess great amount of groupings sulphidrile of basic character [11], then its expected little chromo affinity for the EB.

The staining presented intense staining affinity for deep cells due its abundance of nuclear material; while superficial and mature keratinized cells, had revealed weakly stained for the EB solution.

Conclusion

The cytology had significant improvement in the characterization of the cycle with the technique used in relation the fresh one. It is emphasized chromo affinity of the staining for cells of low maturation, allowing better delimitation of the phases; abundance and cellular conservation to the process of collection, fixation and coloration in liquid-base; praticity and low cost of the technique. The most accurate characterization of the cellular morphology would make possible a quantitative evaluation of the oestral cycle and the establishment of cytological index in a posterior stage of the work.

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RESUMO – Objetivos: O objetivo deste estudo foi à padronização de uma técnica de coleta e coloração em meio líquido que alie a praticidade e a riqueza citológica, possibilitando uma maior reprodutividade e facilidade microscópica. **Métodos**:Ratas wistar (n=20) foram submetidas à coleta vaginal diária em salina e o lavado fixado (éter/álcool) e corado em suspensão com solução de azul de Evans 0,025%. A amostra foi concentrada por centrifugação e observado sob objetiva de 40 x. **Resultados:**Os esfregaços corados permitiram nítida diferenciação das fases do ciclo hormonal (diestro, proestro, estro e metaestro); além da diferenciação dos tipos celulares em relação ao seu grau de maturação tendo como parâmetros o tamanho celular, relação núcleo / citoplasma (RNC) e reação tintorial. O estudo demonstrou a existência de três padrões celulares básicos: células com baixa RNC, acentuada cianofília e pequeno tamanho; células com acréscimo na RNC, perda de cianofilia e maior volume citoplasmático e células queratinizadas anucleadas em aspecto de escama. **Conclusão:** A coloração do material permitiu, além da classificação citológica, a possibilidade de quantificação o que resultaria em um acompanhamento mais acurado do ciclo estral.

DESCRITORES: Epitélio vaginal, ciclo estral, citologia em meio liquido, azul de Evans.

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