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Determination of Oestrous Cycle of the Rats by Direct Examination: How Reliable?

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Summary

For determination of the oestrous cycle in rats classical Papanicolaou technique has long been used successfully. Instead of using many stains in Papanicolaou, staining the vaginal secretions with only methylene blue has also been defined. Recently a new technique in which vaginal samples are directly examined under light microscope has been introduced. The aim of this study was to assess the reliability of this new technique by comparing it with the classical staining techniques. From 20 Wistar rats 60 vaginal samples were collected with a micropipette, three from each. Briefly, the vagina was flushed two to three times then the fluid was placed onto a glass slide. The fluid was equally distributed onto three glass slides. The glass slides were coded. Two samples were stained with Papanicoloau and methylene blue while the other one was examined directly. Determination of the phases of the oestrous cycle was made by the same histologist who was blinded to the groups and coding system. After determination of the oestrous phase in all samples, the results were compared and it was found that the results were matching. In conclusion, the same results can be obtained with the direct examination technique and this technique is reliable, so there is no need to use relatively time-consuming, less practical and more expensive techniques such as Papanicolaou or methylene blue.

Introduction

Animals have long been utilized in experiments that cannot be conducted on humans. Many new drugs or surgical techniques have been tested and as a result new treatment modalities have been developed. Many institutions have experimental animal research centres where extensive research has been carried out and in these centres female rats have been commonly used.

One of the important issues when using female rats in experiments is the determination of the oestrous cycle. For this purpose the classical Papanicolaou technique has long been used successfully (Bancroft and Stevens, 1996). Instead of using many stains in Papanicolaou, staining the vaginal secretions with only methylene blue has also been defined (Bancroft and Stevens, 1996).

Recently a new technique for determination of oestrous cyclicity was introduced in which no stain was used (Marcondes et al., 2002). In this technique vaginal samples were collected with a micropipette and a drop of the vaginal secretion was evaluated under the light microscope. If the same results can be obtained with this technique then there would be no need to use relatively time-consuming, less practical and more expensive techniques such as Papanicolaou or methylene blue.

The aim of this study was to assess the reliability of this new technique by comparing it with the classical staining techniques.

Materials and Methods

Twenty female Wistar albino rats (3 months of age) were used at the Gaziosmanpasa University Experimental Animal Research Laboratory. The rats were caged in a controlled environment with 12-h light/dark cycles and were fed *ad libitum*. The animals were sexually mature and had no sexual intercourse before. The guidelines for the care and use of the animals approved by the local institution were followed. All rats were observed for several days to ascertain the health before sample collection.

Vaginal smears were obtained with a micropipette. Each rat was held at the back of the neck region by an investigator. The head was gently squeezed and fixed with the help of the thumb and the index finger. With the second hand the tail of the rat was pushed aside so that the second investigator could insert the tip of the pipette into the vagina and obtain vaginal smears. The tip of the micropipette was filled with a small amount (one to two drops) of saline (0.9%) and then inserted into the vagina of the female rat. While collecting vaginal lavage samples, being overly aggressive was avoided because the animal could become pseudopregnant which would happen if the micropipette had stimulated the cervix with enough pressure. The vagina was flushed two to three times with the saline and then the fluid was placed onto a glass slide. The fluid was equally distributed onto the glass slides and was spread out with the tip of the micropipette. The glass slides were coded by another investigator. Two samples from the same rat were fixed with alcohol and the third one was left to dry without fixation.

The samples were brought to the Department of Histology where two smears from each rat were stained with Papanicolaou and methylene blue by a histologist who was blinded to the groups and who had no knowledge about the coding system. Papanicolaou and methylene blue staining techniques were performed according to a previously published report (Bancroft and Stevens, 1996).

Determination of the phases of the oestrous cycle was made by the same histologist using a light microscope (40× magni-

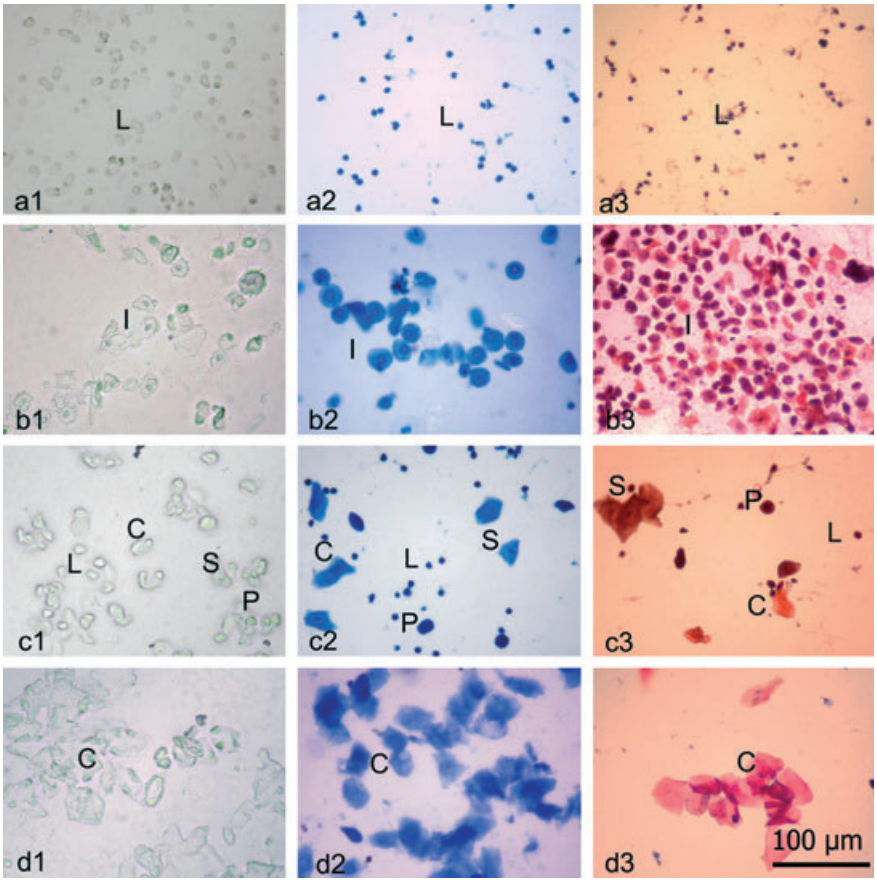

Fig. 1. Photomicrographs of vaginal smears from female rats with three different methods. Dioestrus phase is depicted in a1, a2 and a3, pro-oestrus in b1, b2 and b3, metoestrus in c1, c2 and c3, and oestrus in d1, d2 and d3 with unstained, methylene blue stain and Papanicolaou stain techniques respectively. Leucocytes (L), intermediate cell (I), cornified cell (C), parabasal cell (P) and superficial cell (S) are indicated.

fication, Fig. 1). The cellular types from the vaginal smear of female rats, and the proportion among them help the observer to define the oestrous cycle phase of the rat. The determination was made according to the following criteria. A proestrus smear consists of a predominance of nucleated epithelial cells (Fig. b1, b2 and b3); an oestrous smear primarily consists of anucleated cornified cells (Fig. d1, d2 and d3); a metoestrus smear consists of the same proportion among leucocytes, cornified and nucleated epithelial cells (Fig. c1, c2 and c3), and a dioestrus smear primarily consists of a predominance of leucocytes (Fig. a1, a2 and a3) (Long and Evans, 1922; Mandl, 1951). Determination of the cell types (nucleated epithelial cells, parabasal superficial, intermediate cells) was made according to published data (Bancroft and Stevens, 1996).

Results

A total of 60 slides were prepared, three from each rat. The collection of 60 vaginal secretion materials from 20 female rats took about 60 min. The histologist, who was blinded to the coding system, noted the results of each specimen she had evaluated. For direct unstained observation of the cells it was done with low illumination in the microscope, and without the use of the condenser lens to assure a good contrast.

After evaluation of all the specimens, the coded slides were compared for being in uniformity. All 60 samples had enough quality for evaluation. After determination of the phase of the oestrous cycle in all samples, the results were compared and it was found that the results of all the three samples from each rat were matching. Six rats were found to be in oestrus, six were in dioestrus, four were in pro-oestrus and four were in metoestrus phase with the three methods. Examples of each phase of the oestrous cycle with different determination methods are depicted in Fig. 1.

Discussion

Most assessment strategies for assigning the oestrous stage of rats rely on the relatively noninvasive technique of examining vaginal smears at 24 h intervals. The oestrous cycle of the rats lasts 4 days and is characterized as proestrus, oestrus, metoestrus and dioestrus, which may be determined according to the cell types observed in the vaginal smear (Long and Evans, 1922; Mandl, 1951; Freeman, 1988). There are several strategies for assessment of these cell types: staining methods, Papanicolaou and staining with methylene blue, and direct examination method which has gained acceptance recently as it is proposed to be faster and more practical (Marcondes et al., 2002). In the present study the reliability of the direct examination technique was assessed by comparing it with the classical staining techniques, and it was found that the results from the three techniques matched exactly.

Papanicolaou staining is the classical method that has been used to evaluate cellular changes in vaginal cytology. In this method the specimen must be treated with graded concentrated alcohol solutions first, and then orange G 6, EA50 and Harris haematoxylin solutions are used for staining. Thereafter, the specimen is again treated with graded concentrated alcohol and is placed in xylene for 15 min before covering with a cover glass. The collection of vaginal secretion and the use of

stained material generally take 1–2 h or more. In this study another staining method, methylene blue staining, was used. In this method the specimens are treated with 1% methylene blue for 45 min. The specimens can be examined after they dry. All these procedures take about 1 h or more.

In the direct examination method, unstained native material can be observed using the microscope without need to wait for the material to dry. This method is suggested to offer a fast and practical way to determine the phases of the oestrous cycle in the rat (Marcondes et al., 2002). It is suggested to be useful in long protocols in which the determination of the phases of the oestrous cycle is made for the experiments that last some hours or all day (Rodrigues et al., 1995; Chateau et al., 1996; Marcondes et al., 1996; Vanderlei et al., 1996; Spadari-Bratfisch et al., 1999). In addition, the lower expenditure – no need for staining – is another concern. However, the reliability of this method has not been assessed by comparing it with other staining methods.

One issue that has to be considered in interpreting these data is stimulation of the vaginocervical area of the rats by the micropipette. If the micropipette is aggressively inserted, it stimulates the cervix, inducing a neuroendocrine reflex that can cause pseudopregnancy and, therefore, disrupt the rat's oestrous cycle for approximately 14 days (Gunnet and Freeman, 1983; Steuer et al., 1987). In the present study the rate of pseudopregnancy occurrence with micropipette was not studied; therefore, if this technique is to be used for determination of the phases of the oestrous cycle, it must be kept in mind that being overly aggressive can cause pseudopregnancy. Further studies that assess the rate of pseudopregnancy occurrence with this method would help clarify this issue.

In the present study the reliability of the direct examination method was assessed by comparing the results with the other classical methods and it was found that the results were exactly in accordance with Papanicolaou and methylene blue staining methods. From this point, for evaluation of the cellular changes in vaginal cytology, direct examination under the light microscope seems to be a reliable method and may be the preferred one as it is fast, practical and less expensive.

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