Original Research Article

Journal of Biological Research

& Biotechnology

Bio-Research Vol. 20 No.2; pp. 1600-1605 (2022). ISSN (print):1596-7409; eISSN (online):2705-3822

The effects of variations in length of suction pipette inserted during vaginal smear cytology procedure on estrous cycle in Sprague–Dawley rats

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Abstract

Estrous cycle is a periodic reproductive sequence of events in most female laboratory animals that are used for basic research. Several methods have been used to stage estrous cycle, these include visual method, vagina smear cytology evaluation, chemical analysis of urine and the analysis of electrical resistance. The vaginal smear cytology (VSC) evaluation method is commonly used to stage estrous cycle because it is a faster method coupled with easy execution and not as expensive as the other methods. The study of estrous cycle is primary to all investigations in reproduction and related areas. For the generation of dependable research outcome from VSC evaluation method, the technique for the collection of vagina smear and its microscopic interpretation must be done according to established standards. Many reports in literature concerning VSC evaluation method, lack comprehensive demonstrations and uniformity which lead to loss of homogeneity and hence provide study outcomes that are not authentic. Therefore, this present investigation was designed to establish the standard value of inserted length of suction pipette during smear extraction technique when using VSC method to study estrous cycle. A total of fifteen experimental animals were recruited for this investigation. The animals were subdivided into three groups 1 to 3. The lengths of pipette inserted into the vagina canal were varied from 1mm, 2mm to 4mm in group 1, 2 and 3 respectively. The outcome from this investigation showed that the depth of suction pipette inserted during smear extraction procedure in VSC method has effect on estrous cycling. Estrous cycle was maintained with 2mm insertion length with approximate four days cycle and phases changing daily.

Keywords: Estrous, Vagina, Smear, Pipette, Length, Cytology

Received Jan 25,2022; **Revised** May 17, 2022; **Accepted** June 2, 2022

https://dx.doi.org/10.4314/br.v20i2.7
Journal Homepage: **http://www.bioresearch.com.ng.**
Publisher***: Faculty of Biological Sciences, University of Nigeria, Nsukka, Nigeria.***

INTRODUCTION

Estrous cycle is a rhythmic physiological action in female animals that is controlled by hormones. This is synonymous with menstrual cycle in humans (Geoffrey *et al.,* 2007, Eder *et al.,* 2020). The use of female mammals in basic medical research is gradually being excluded due to the apparent strain caused by the study of estrous cycle. Obviously, the significance of gender dissimilarity in varieties of reproductive and neurological conditions due to variations in hormone regulations, investigations done on female laboratory animals remain critical and important to scientific enquiries (Trishala *et al.,* 2020). There are several methods through which estrous cycle can be studied; these include visual method, electrical resistance analysis, vagina smear cytology (VSC) evaluation and chemical analysis of urine (Gnanagurudasan *et al.,* 2017, Robert *et al.,* 2021). VSC evaluation method is generally used because it is faster to execute, can easily be carried out and it is not as expensive as other methods. More importantly, VSC method is ideal when all the phases of estrous cycle are needed to be establish which is conventional (Shannon *et al*., 2012, Bakare *et al.,* 2021). The cytological analysis of different cellular components of extracted vagina smear is the basic concept of VSC method. The extracted smear is gently dropped onto a glass slide and covered with a coverslip. This is then viewed under the microscope at 40X objective lens magnification for cellular analysis. The microscopic analysis of extracted smear is done by using cell types and population to characterize the phases of estrous cycle (Marcondes *et al.,* 2002, Hubscher *et al.,* 2005). The study of estrous cycle is primary to most female reproductive investigations done in basic medical laboratories. The study of estrous cycle is preliminary to investigations on pregnancy, ovulation, reproductive hormone profile analysis, uterine parameters, fertility evaluation and other investigations that are related to reproduction. To generate authentic and reliable data from VSC method, smear collection technique and its histological evaluation must be based on well-established standard principles (Michelle *et al.,* 2015). Several reports from literature regarding VSC method are not specific and consistent (Michelle *et al.,* 2015). The insertion length of the suction pipette, quantity of extracted smear and number of smear spots created on glass slide are not consistent in the studies that were reported in literature (Long and Evans, 1922, Marcondes *et al.,* 2002, Hubscher *et al.,*2005, Caligioni, 2009, Ayodeji and Roland, 2020).

During VSC procedure, the length of pipette inserted by an author was 2 mm in mice (Michelle *et al.,* 2015). Inserted length of 4mm was also reported by another author (Marcondes *et al.,* 2002), while in some references, the insertion lengths were not specified. Pseudo-pregnancy is the occurrence pregnancy symptoms in the absence of gestation. It is most common in female mammals particularly in rodents, a readily available choice of model for basic scientific investigation.

The continuous striking of the vagina wall has been reported to induce pseudo-pregnancy. Pseudo-pregnancy is induced because of vaginal wall sensitization due to strike action of the suction pipette where animals sense the pipette as the penis (Kaneko *et al.,* 2020). This may induce pseudo-pregnancy and consequently disrupt estrous cycle. This action may cause erroneous research conclusions as effect of test substance on estrous cycle in laboratory investigations may be due to the induction of pseudo-pregnancy and not the true effect of the test substance. Therefore, it is imperative to establish standard value in-terms of the inserted length of pipette into the vagina canal during VSC procedure. This will eliminate interference on research outcomes as the striking action of the wall of the vagina canal may induce acyclicity through the induction of pseudo-pregnancy or other indefinite activities that may disrupt estrous cycle. Clearly, this study will address the problem of inconsistencies and unspecified practices in VSC procedure as it relates to the inserted length of suction pipette. Furthermore, this present study will add to research knowledge and protocol in creating a more effective skill of VSC method of studying estrous cycle that will provide research findings that are authentic and genuine.

MATRIALS AND METHODS

Animal model, population, source, and maintenance

A total of fifteen cyclic female Sprague-Dawley rats were used for this experiment. The animals were obtained from animal breeding stock, Yaba, Lagos. The rats were kept under standard laboratory conditions and allowed to acclimatize in the research laboratory for two weeks. All procedures involving animal use for research conform to the guiding principles as recommended by standard practices (American Physiological Society, 2002).

Animal distribution, events, and durations

The animals were divided into three experimental study groups 1 to 3. In study group 1, 2 and 3, the inserted lengths of pipette into the vagina canal were 1mm, 2mm and 4mm respectively for 20 days.

Table 1. Staging of estrous cycle in the experimental animals

Group	Population	Length of pipette inserted (mm)
1	5	1.0
2	5	2.0
3	5	4.0

Smear extraction technique

Estrous cycle is determined by day-to-day microscopic interpretation of vagina smear done at specific time in the morning and this was maintained throughout the course of the experiment. The rat was held in place with one hand around its waist with the ventral surface downward for support whilst the other hand was used to hold the pipette. A small amount of normal saline about 0.2 ml was first sucked up into the pipette. This was then inserted into the vagina canal at varied lengths to collect the smear. The normal saline was released into the vagina and sucked back up into the pipette. The collected smear was then dropped onto a clean glass slide, covered with a cover slip to ensure that the collected smear is in uniform orientation, making it easier to focus and to avoid smear coalescing during movement. The smear was then viewed under the microscope with 40X magnification (Marcondes *et al*., 2002, Ayodeji and Roland, 2020).

Microscopic analysis of collected vagina smear

Estrous cycle lasts an average of four days in rats, and it is characterized by four progressive phases changing from the estrus, proestrus, diestrus to metestrus. The changes within the internal lining of the vagina wall due to the actions of hormones are used as a valuable marker for staging estrous cycle. Therefore, the presence, absence, or population of cornified cells and leucocytes were used in establishing the phases of estrous cycle. The first phase of the estrous cycle was designated as the metestrus, the presence of leukocytes amidst remnants of large squamous cells in the smear histology was used to stage this phase. The second day was termed the diestrus phase with smear presentation of small, nucleated cells when viewed under the microscope. The third day of smear histology seen with numerous large, nucleated cells was marked as the proestrus phase. The proestrus phase of estrous cycle marks the maturation of ovarian follicles and the initiation of ovulation. The fourth day was designated the estrous phase with histological evaluation of large flakes of squamous cells. This phase marks the stage when the animals were on heat and most receptive to male partners (Marcondes *et al.,* 2002).

Statistical Analysis

Data obtained from all groups were compiled and analyzed using One-way Anova. Statistical significance was determined by Turkey post hoc test and considered at $p < 0.05$.

RESULT

The normal cycling pattern of approximately four days with estrous phases changing progressively was seen in group 2 where 2mm of suction pipette was inserted into the vagina canal, this showed estrous cycle of an average of four days with completed five estrous cycles in the 20 days experimental duration with phases changing from metestrus, diestrous, proestrus to estrous (Figure 2). The cycling pattern seen in the 1mm insertion length in group 1 with established estrous cycle of an average of ten days with completed two estrous cycles for the 20 days duration was abnormal. Additionally, the 4mm insertion length in group 3 showed estrous cycle of an average of five days with completed estrous cycle of four days (Figure 2) which was also not the normal cycling pattern in rats. However, 2mm insertion length showed the normal cycling rate with an average of four days with approximately five cycles completed as expected in the twenty-day duration. Even though the diestrus phase is the longest phase of estrous cycle, the number of days spent on diestrus phase was abnormal in group 1 and 3 with the highest number of days in group 1 followed with group 3 for approximately fifteen and thirteen days respectively. The animals spent more than half of the experimental duration of 20 days on the diestrous phase (Figure 1). The animals in group 2 established a statistically significant lower number of days spent on diestrous phase when compared to other groups (Figure 1). The animals in group 2 showed normal transition from diestrus to estrus phase of estrous cycle. More so, the number of days spent on estrous phase when animals were on

heat was statistically significant lower in group 1 and 3 (Figure 1).

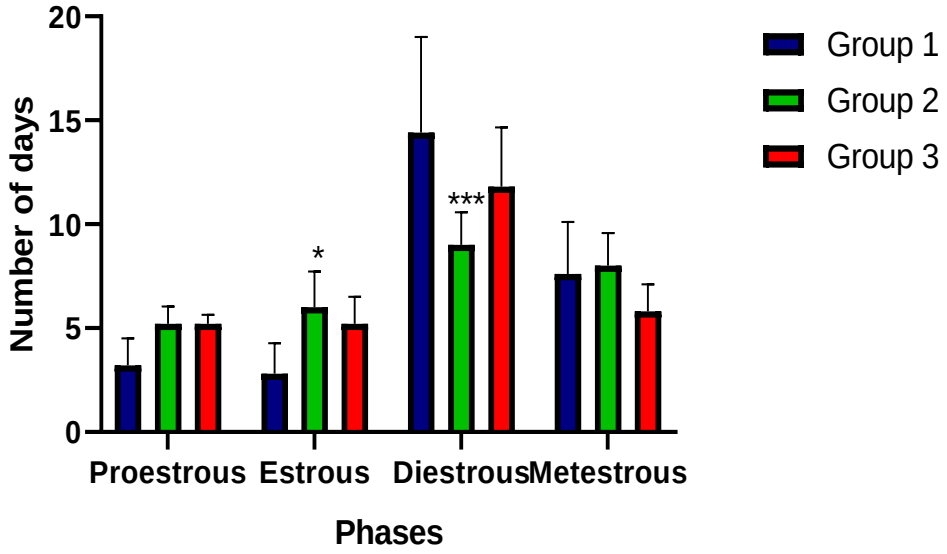

FIGURE 1: Number of days spent on each estrous phase after 20 days duration

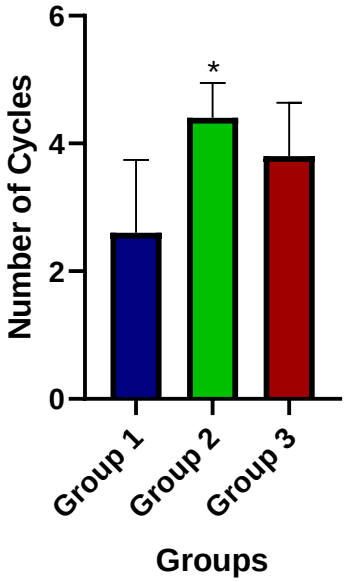

FIGURE 2: Number of completed estrous cycles after 20-day duration

DISCUSSION

Many studies in clinical and basic sciences indicate that modifications of experimental protocols provide opportunity for improvement and better research outcomes. Most female mammals undergo estrous cycle as compared to menstrual cycle in human. In estrous cycle, the phases change daily from one phase of estrous cycle to the other. This is influenced by reproductive hormones where each phase of estrous cycle is established by specific hormone activity and

last for definite duration of time (Marcondes *et al*., 2002, Ayodeji and Roland, 2020).

The development of corpus luteum which occurs due to decrease in progesterone level in the absence of implantation is the key event of the metestrus phase. This phase is short and may last for about seven hours in rats (McCracken *et al.,* 1999). The next phase is the diestrus phase and it is characterized by continuous decrease in progesterone secretion which causes the inhibition of luteinizing hormone and consequently leads to a functional degeneration of corpus luteum at late diestrus. The diestrus phase occurs with the longest period ranging from fifty-five to fifty-seven hours (Spornitz *et al.,* 1999). The proestrus phase is the next phase which features increase in follicular growth. This is stimulated by follicle stimulating hormone (FSH) and luteinizing hormone (LH) under the activation of estrogen in a positive feedback manner. Once follicles are fully developed, reduction of FSH and LH secretion occur. This is followed with the initiation of egg release in late proestrus after an abrupt spike in LH level. The proestrus phase usually last for about thirteen hours in rats (Caligion, 2009). The last phase of estrous cycle is the estrus phase where the uterine endometrium starts to develop for implantation process. This is influenced by estrogen and progesterone and lasts for about twenty-six hours (Geoffrey, 2007).

Furthermore, there is a perceived manipulation of hormone due to the striking action of the wall of the vagina during smear extraction procedure in VSC evaluation method. The hormones associated with pregnancy may be activated due to continuous striking of the vagina wall to initiate false pregnancy (Kaneko *et al.,* 2020). Therefore, the inserted length of pipette is apparently a critical factor in maintaining estrous cycle. The outcomes of this present study showed that variation in the lengths of suction pipette inserted into the vagina during VSC method affect estrous cycling pattern. The animals inserted with lengths of 1mm and 4mm spent more days on diestrus phase when compared to the 2mm insertion length (Figure 1). The 2mm insertion maintained normal cycling rate of approximately 5 cycles in 20 days while insertion of 1mm and 4mm presented cycling rates of approximately 2 cycles and 4cycles respectively in 20 days respectively (Figure 2). These outcomes showed that insertion at 2mm maintained cycling in the laboratory animals and infers that the suction pipette did not alter hormone secretion pattern of the animals. However, the suction pipette inserted at 4mm may have triggered reproductive hormone activations in response to the striking action of the wall of the vagina. Additionally, the insertion of 1mm may present a sharp, intense and more direct striking effect on the vagina wall that may have induced pseudo-pregnancy (Ayodeji and Roland, 2020, Kaneko *et al.,* 2020).

CONCLUSION

It is evident that the depth of suction pipette inserted during smear extraction technique in VSC method of studying estrous has effect on estrous cycling pattern. The best cycling rate was established with 2mm insertion length with normal number of completed estrous cycles and days spent on each phase of estrous cycle. Therefore, the 2mm insertion length is recommended for use during vagina smear cytology method of studying estrous cycle in rat.

Conflict of interest

Authors have no conflict of interest to declare

Author contributions

BAA: project execution, result analysis and manuscript writing. ICA: result analysis and manuscript writing. SZA: project execution. AF: result analysis and manuscript writing.

Acknowledgement

We appreciate the faculty of Basic Medical Sciences, University of Lagos for providing the fund for the execution of this study (FBMS NO: 2018/10).

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