ORIGINAL ARTICLE

ASSESSMENT OF VAGINAL SMEAR CYTOLOGY PROCEDURE IN SOUTH WESTERN NIGERIA INSTITUTIONS

Running Title: Vaginal Smear procedure in Niegria

ABSTRACT

Estrous cycle is the main reproductive cycle of female mammals that are used for basic laboratory investigations. Vaginal smear cytology (VSC) is the most common method used in studying estrous cycle. There are multiple conflicting references regarding the VSC procedure, many of which lack comprehensive illustrations with undesired outcomes. These disparities have been linked to variations in environmental factors, hence the call for a region driven protocol development and modification. Specifically, activities such as; the length of suction pipette that is inserted into the vagina, the volume of collected smear and the pattern of animal grouping are not consistently stated in the available references. These activities are subject to influencing estrous cyclicity even before the administration of test substance; thereby causing research results that are not authentic. This assessment is therefore aimed at evaluating the procedure, factors that are being considered and result outcome.

The assessment was carried out using questionnaires administered to basic scientists in three south western Nigeria Institutions; University of Lagos, University of Ibadan and Ladoke Akintola University.

The results from this study established that the length of the suction pipette inserted and the quantity of smear volume collected during VSC procedure were always measured and maintained in only 27% of respondents, sometimes in 64% and never in 9%. More so, researchers do not follow specific pattern of grouping where age and weight based grouping pattern are higher with 47.2% and 48.0% respectively than using estrous cycle phase with 4.8% and no basis with 0.3%. The evaluation on result outcome showed that effective cycling at 100% is the lowest while cycling rate at 50% is the highest.

We therefore conclude that the evaluations from these results and that of the available references be put into test to establish a standard model for usage in this region of the world.

Key words: Estrous cycle, Vagina, Smear, Cytology Procedure

INTRODUCTION

Estrous cycle is the main reproductive cycle of female mammals used for basic laboratory investigations such as; rats and mice. The estrous cycle is synonymous with menstrual cycle in human. The estrous animals reabsorb their endometrium in the absence of pregnancy which is in contrast with the menstrual cycle in which the endometrial lining is shed through menstruation

(Susan et *al.* 2004; Geoffrey et *al.* 2007). The estrous cycle comprises of recurring physiologic and behavioral changes that are induced by reproductive hormones (Kaiyu *et al.* 2016). Therefore, the study of estrous cycle has been used to measure the integrity of the hypothalamic-pituitary-ovarian reproductive axis and also formed the preliminary of most reproductive-based investigations which include; ovulation, uterine parameters, mating and fertility evaluation (Justin and Adam, 2018; Kiandokht et *al.* 2018). Estrous mammals can be monoestrous, diestrous or polyestreous. Monoestrous species, such as; bears, foxes, wolves and white-tailed deer undergo estrus once in a year while diestrous animal (dog) goes into estrus twice per year. Polyestrous species such as; rodents, cats, cows and pigs go through a succession of estrous cycles during the year (Junko et *al.*, 2016). The length of estrous cycle varies among species, but typically more frequent in smaller animals (Perera, 2011). The estrous cycle in rats and mice is short and precise; this makes them ideal for basic laboratory investigations. They undergo periodic changes from proestrus, estrus, metestrus to diestrus phase within 4 to 5 days (Michelle *et al.* 2015).

The study of estrous cycle can be done using a variety of methods such as; visual method, electrical impedance method, vagina smear cytology and biochemical analysis of urine. The vaginal smear cytology (VSC) method is the most common and has long been used to study estrous cycle because it is faster, easier and less expensive when compared with other methods. More so, it is preferred when all stages of the estrous cycle are needed to be identified (Shannon *et al.* 2012). The other methods such as; the visual method, measuring electrical impedance and biochemical analysis of urine involve more technical protocol and/or do not provide information on all the phases of estrous cycle, hence are not commonly used (Gnanagurudasan *et al.* 2017). The VSC procedure basically entails the collection of vagina smear with the suction pipette, followed with the histological analysis of the smear. The vaginal epithelium changes in response

to sex hormone at different phases and this is used as the basis for determining the phase of estrous cycle. The collected smear is transferred onto a dry glass slide, stained, overlaid with a cover slip and viewed under a magnified bright field illumination. The cytological analysis is done by using the morphology of smear epithelium to characterize the different phases of estrous cycle which is labeled as the proestrus, estrus phase, metestrus and diestrus (Marcondes *et al.* 2002; Cora et *al.* 2015).

The proestrus phase is the first phase of estrous cycle and it is regulated by gonadotropic hormones which stimulate the maturation of ovarian follicles. The vaginal smear histological appearance on this phase shows the presence of sheets or isolated nucleated epithelial cells and cornified cells. The duration of proestrus phase in rat is usually about twelve to fourteen hours (Camila et *al.* 2013). The estrus phase refers to the period when the female is sexually receptive "in heat" or "on heat" to the opposite sex. The vaginal smear shows primarily non-nucleated cornified cells. Fertile mating on estrus phase of the cycle leads to pregnancy, but infertile mating leads to a state of pseudo-pregnancy. Estrus phase lasts for about twenty-five to twenty-seven hours. The vagina smear histology during the metestrus phase shows leukocytes, a few cornified and basophilic cells. This phase is typically brief and last for six to eight hours in rats (Cora et *al.* 2015). The diestrus phase is characterized by the activity of the corpus luteum that produces progesterone. The vaginal smear histological appearance is characterized by little mucus with some leukocytes, nucleated basophilic cells and occasionally vacuolated cells. The diestrus occurs with the longest period of time which is usually between fifty-five to fifty-seven hours (Marcondes *et al.* 2002; Paccola1 *et al.* 2013).

There are multiple references regarding this procedure, many of which lack comprehensive illustrations and specificity. These have lead to loss of consistency and undesired research outcomes (Cora et *al.* 2015). The techniques involved in VSC procedure such as; the length of

the suction pipette inserted into the vagina canal, volume of extracted smear and basis for grouping animals are not specific and consistent within the context of the available multiple references from the earliest to recent (Long and Evans 1922; Marcondes *et al*. 2002; Byers *et al.* 2012). More so, the aforementioned parameters are subject to causing pseudocyesis and also interfering with the vagina micro-environment. The psychology of the animals could as well be affected, thus capable of influencing cycling pattern even before the administration of test substance, thereby giving research results that are not authentic.

Pseudocyesis or false pregnancy is the appearance of signs and symptoms associated with pregnancy when the organism is not actually pregnant. It occurs as a result of hormonal imbalances translating into physical changes similar to those of pregnancy (Amanda et *al*. 2018). It has been reported from literature that continuous striking of the vagina wall induced stress and pseudo-pregnancy in laboratory animals and consequently disrupts estrous cycle (Fox, 2007). The striking effect maybe generated as a result of smear extraction technique. The animal senses the suction pipette as penis thereby causing false mating which consequently leads to pseudopregnacy. Therefore, the length of the suction pipette inserted during VSC procedure may be one of the aspects of VSC procedure that needs to be established. More so, reproductive function and estrous cycling have been shown to be sensitive to changes in vagina microenvironment which could result from intrusion into the vagina cavity and extraction of vagina smear (Aiko et *al*. 2017). Furthermore, the basis onto which animals are grouped needs to be ascertained as animals on different phases exhibit different psychological behavior which may induce stress on other animals in the same group and consequently affect their cycling pattern (Geoffrey *et al.* 2007).

In summary, these events can generate false research outcomes where research results are basically generated as a result of inefficiencies and not from true effects of test substance.

Indeed, the length of suction pipette inserted, volume of smear collected and pattern of animal grouping during VSC procedure are parameters that could disrupt estrous cycle. It is therefore imperative to assess this procedure to establish the areas of challenge in the quest of establishing standards in a subsequent modification study.

Therefore, this study will establish a direction through which a modification protocol will be directed using the following specific objectives:

i. To evaluate if the length of suction pipette inserted during VSC procedure is measured and maintained.
ii. To evaluate if the volume of smear extracted during VSC procedure is measured and maintained.
iii. To determine the basis for animal grouping during VSC- based investigations.

MATERIALS AND METHODS

Approach

This assessment study utilized questionnaire-based assessment approach. The study was carried out using short questionnaire format to provide an individual's opinion and practice of VSC procedure to generate reliable and valid inferences (Salisbury *et al*. 2005). Undergraduate and postgraduate research students and faculty members in basic medical faculties of three south-western Nigeria Universities; University of Lagos, University of Ibadan and Ladoke Akintola University of Technology were employed to complete the questionnaires.

Design and Administration of Questionnaires

The questionnaire consists of four sections that addressed the study objectives. Section A determined the backgrounds of the respondents while section B confirmed their knowledge and practice of the VSC procedure. Section C of the questionnaire provided answers to the study objectives. This section provided answers to factors such as; the length of suction pipette inserted

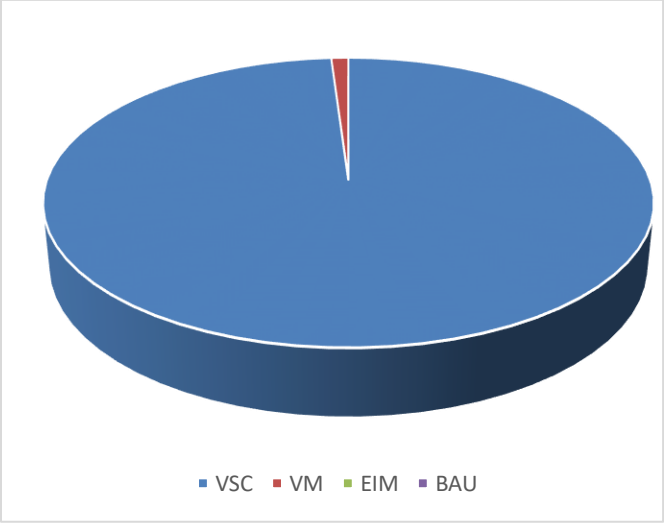
into the vagina, volume of extracted smear, number of smear spots created on glass slide and basis for animal grouping. The last Section which is the D section assessed the cycling rate during VSC-based study. The administrations of questionnaires were done for over a six month period for the three institutions. The questionnaires were administered to researchers who have prior and current experience in VSC-based investigations. A total of 300 researchers from the three institutions completed the questionnaires. All the sections in the questionnaires were filled and collated for analysis.

Statistical Analysis

The data collected were analyzed using Microsoft excel version 16.0 and results were interpreted using pie chart graphs.

RESULTS AND DISCUSSION

It was observed that most researchers reached were interested in filling the questionnaire as they claimed that the VSC procedure comes with a lot of challenges. This study showed that the VSC method of studying estrous cycle is the most commonly used with the highest percentage (Figure 1).



VSC- Vagina Smear Cytology
VM- Visual Method
EIM- Electrical Impedance Method
BAU- Biochemical Analysis of Urine

Figure 1: showing the different methods used in studying estrous cycle

The results on the measurement and maintenance of the length of suction pipette inserted and the quantity of smear volume collected were always measured and maintained in only 27% of respondents, sometimes in 64% and never in 9%. The respondents who ascertained that the length of pipette measured and maintained did not measure in standard values but gagged and maintained (Figure 2).

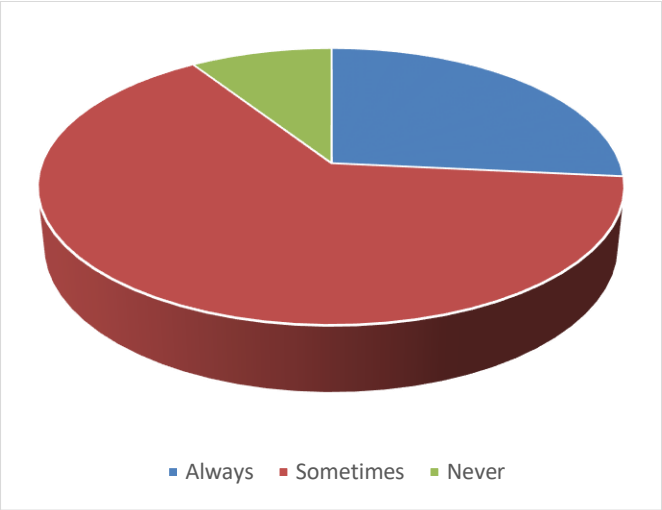


Figure 2: showing the measurement and maintenance of the length suction pipette inserted and the quantity of smear volume collected

The result on grouping pattern showed that the researchers do not follow a particular pattern of grouping during estrous cycle study where age and weight based grouping pattern are higher with 47.2% and 48.0% respectively than using estrous phase with 4.8% and no basis with 0.3% (Figure 3).

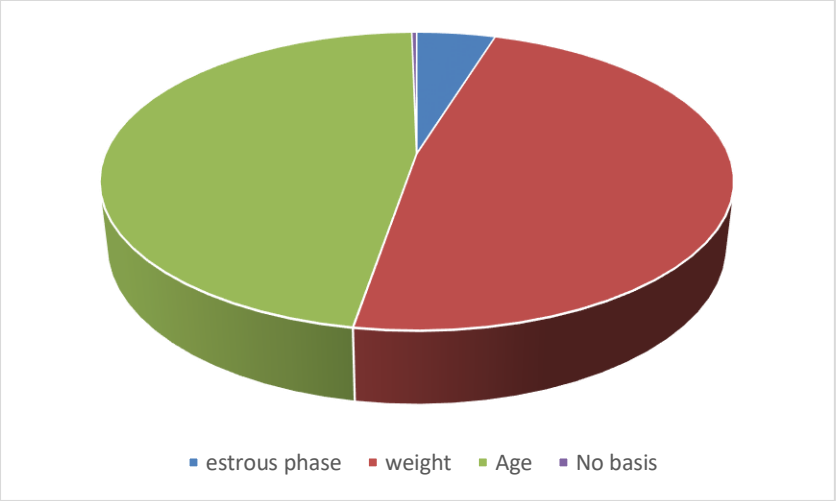


Figure 3: showing the pattern of animal grouping

The evaluation on result outcome from figure 4 showed that effective cycling at 100% was found to be lowest while cycling rate at 50% was the highest. This means that half of a group of study animals maintained their estrous cycle while the remaining animals refused to cycle. Therefore, it is possible that some aspects of the VSC procedure may have generated into cycling inhibiting factors.

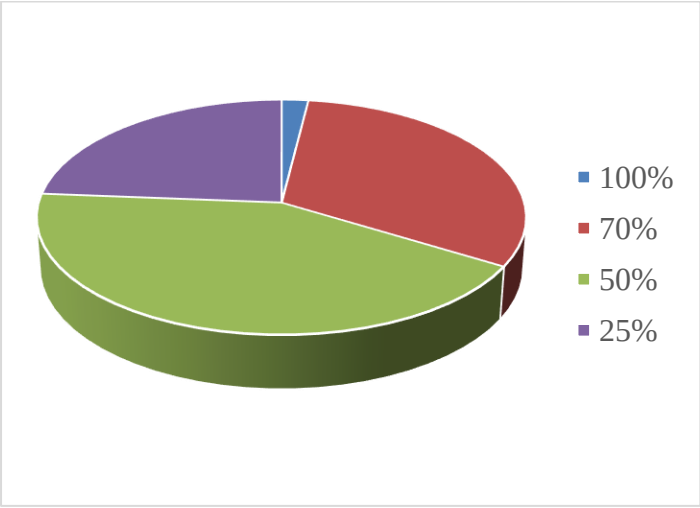


Figure 4: showing Estrous Cycling Rate

More so, respondents reported that animal's refusal to cycle was noticed most after the second completed estrous cycle (62%) while after the first (18%) and third (19%) estrous followed with 4th cycle (1%) (Figure 5).

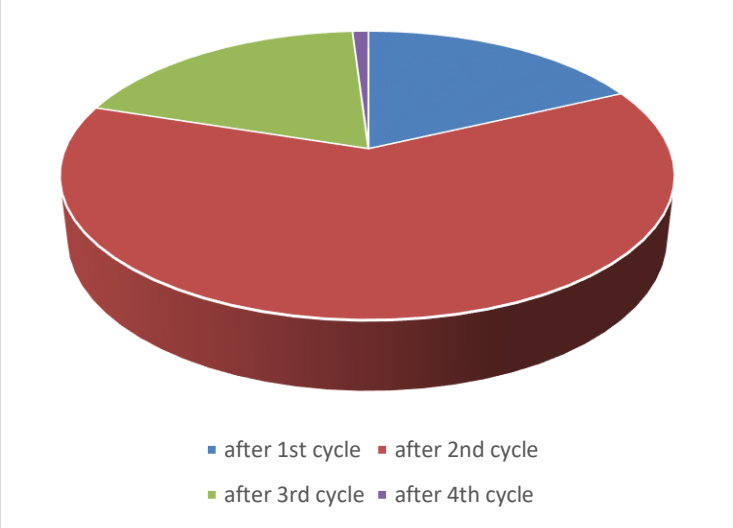


Figure 5: showing the commencement of acycling

DISCUSSION

The result from this study further confirmed that VSC procedure as the most common method used for studying estrous cycle (Shannon et *al.* 2012). It is evident that researchers are not completely satisfied with this procedure. There are a number of studies done to modify certain aspects of the VSC procedure. In a previous modification study conducted on different device used in extracting vagina smear, the eye dropper and a cotton bud compared with the suction pipette. The different devices were investigated on the overall simplicity and easiness of the procedure. The modified suction pipette group demonstrated a faster and simpler way of extraction and evaluation of extracted smear than the other devices. The other device techniques may induce stress on the tested animals and affect the quality of vagina smear as the obtained

smear appeared unclear due to cells overlap (Mahfoudh, 2016). However, the length of the suction pipette inserted during the procedure is also important. The standard length of suction pipette inserted during VSC procedure needs to be established in standard value as continuous and intense striking of the wall of the vagina may lead to pseudocyesis and consequently inhibit cycling. Another study was done to standardize staining for cytological richness and microscopic easiness. This study compared daily vaginal smear collection in saline and a solution of Evans Blue 0.025%. The samples were pondered by centrifugation and observed under lens of 40X. The stained smears allowed clear differentiation of the phases through the differentiation of the cellular types and sizes. The stained smear allowed clearer cytological classification and quantification than the former (Rand, 2005). The basis for animal grouping during VSC procedure calls for attention. The result from this evaluation shows that age and weight of animals are mostly used for grouping. Meanwhile animals on different phases of estrous tend to behave differently because of hormone influence. It has been reported that animals on estrus phase of estrous cycle are hyperactive and may infringe stress and consequently affect their cycling pattern of others animals when grouped together (Geoffrey *et al.* 2007). It is evident that a more reliable and consistent data can be generated from vaginal cytology studies, if procedure is based on well-established standardized criteria (Michelle, 2015). Therefore standards should be put to the various aspect of VSC procedure as the most common and easiest method of studying estrous cycle in laboratory animals to generate research findings that are more genuine and authentic. Following this assessment study which is basically to provide adequate information and guide needed to understand the challenges experienced, a subsequent modification study will be carried to develop a functional protocol for VSC procedure for researchers in this part of the world.

CONCLUSION

Indeed, challenges emanate from the VSC procedure which is needed to be established before a modification. This assessment evaluation projects the basis for a subsequent modification study which will be used to establish standard protocols for VSC procedure for scientists in this region of the world. We therefore, recommend that the outcome of this assessment with the different variations stated in the available references be put into test to establish a model for usage.

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