VAGINAL CYTOLOGY IN BUFFALOES: A REVIEW

Araya Suebkhampet[1,*] and Thuchadaporn Chaikhun Marcou[2]

ABSTRACT

Vaginal cytology is a simple technique that can be used to determine the stages of the estrus cycle, if there are pathological conditions of the reproductive tract, and the optimal time of mating. There have been many vaginal cytology studies and it's clinical applications have been reported in many species. There have been few reports, however, about its use in buffalo. Buffaloes are considered poor breeders as their reproductive efficiency is adversely affected by certain limitations such as: late maturity, seasonality of breeding, silent heat coupled with poor expression of estrus, low conception rates and long intercalving period. Accurate heat detection is one of the key factors to increase the conception rate. Vaginal cytology in combination with other heat detection methods can help in this process.

The objective of this review article is to focus on the use of the vaginal cytology technique for estrus detection and other applications in buffalo.

Keywords: vaginal cytology, vaginal smears, buffaloes, *Bubalus bubalis*, estrus cycle

INTRODUCTION

Buffalo populations and buffalo farming businesses have been increasing in every country, including Thailand (Department of Livestock Development, 2018). Buffalo dairy and meat products are in high demand by consumers both as traditional food and as functional health food. However, buffalo productivity is limited by the species' reproductive characteristics such as a late age of puberty, a long gestation period, and difficult heat detection due to a "silent" (hard to detect) heat (De Rensis *et al*., 2005; Chaikhun *et al*., 2010). These issues can cause a long calving interval and reduce the lifetime productivity of the animal.

Artificial insemination (AI) is frequently used for genetic improvement and breeding management nowadays. Heat detection, however, is key to getting a high pregnancy rate from AI. Farmer's breeding protocols are variable: direct estrous signs observation, heat detection instruments and hormonal treatments with fixed time AI are just some of the methods employed. The costs, skills required and the accessibility of each technique vary.

Vaginal cytology is known to be a quick

[1]Pre-Clinical Veterinary Science Department, Faculty of Veterinary Medicine, Mahanakorn University of Technology, Bangkok, Thailand, *E-mail: saraya@mut.ac.th, anupriwan@yahoo.com

[2]Obstetric Gynecology Andrology and Artificial Insemination in Domestic Animal Clinic, Faculty of Veterinary Medicine, Mahanakorn University of Technology, Bangkok, Thailand

and effective technique for the evaluation of the optimal mating time and estrous cycle for dogs (Fay *et al*., 2003). It has also recently been tested and successfully used for estrous cycle evaluation and Artificial Insemination (AI) timing in buffaloes (Duran *et al*., 2015; Chaikhun *et al*., 2008). So far, however, information regarding vaginal cytology techniques for reproductive determination in buffalo has been limited. General information, basic and specific techniques, and possible directions for future study and use of vaginal cytology in buffalo are provided in this review article.

VAGINAL CYTOLOGY TECHNIQUE

The vaginal cytology technique used in buffaloes is similar to that of other mammalian species, including cows. Although minimal - there are differences. The details are as follows. The vulva should be cleaned with tap water and dried with a clean cloth or tissue paper before swabbing. This is done in order to avoid dirt and other contamination in the cell smears. A vaginal speculum or vaginoscope is then used to locate the vaginal position. After this, a sterile cotton swab (ten inches long) moistened with sterile water (if needed) is inserted into the vaginal opening to a depth of about 5 to 8 inches, depending on the buffalo's anatomy (Figure 1A). Vaginal cells are then collected from the dorsal part of the vagina, just before the external os of the cervix. The cotton swab should be rotated at least 90 to 180 degrees against the vaginal wall and then withdrawn gently (Duran *et al*., 2015). The smears are prepared by rolling the swabs onto glass slides with minimal pressure to avoid cell rupturing (Figure 1B). The smears are dried in room temperature for 10 minutes then immediately fixed by soaking in absolute methanol or Carnoyl fixative. After air drying, the smears are stained with Geimsa stain for 30 to 45 minutes. This produces good staining. Glass slides are then rinsed with water and dried. In addition, Leishman or Diff-Quick stain can be used for the staining (Duran *et al*., 2015; Chaikhun *et al*., 2008; Ola *et al*., 2006). Diff-Quick stain may be convenient for field work staining since it can be done by making easy, fast protocols with a good stain quality (Figure 1C to D). In general, Romanowsky-type stains typically used for blood films provide good morphologic identification. The particular fixation and staining protocols should be performed according to the manufacturer instructions.

Observations are done using a light microscope with magnification of 100X for slide scanning and 400X/1000X for particular cell identification. At least 200 vagina epithelial cells per slide should be counted and classified. Differential cell counts are expressed as a percentage. There are many cytological indices such as the cornification index, the carriopyknotic index, the superficial cell index and the maturation index which are utilized for different stages of the estrus cycle (Felipe *et al*., 2001).

VAGINAL CYTOLOGY

Classification of vagina epithelial cells

In mammals, the vaginal epithelium undergoes a predictable hyperplastic response to increasing plasma estrogen during proestrus. Starting as only a few cell layers, the epithelium becomes many layers and causes a thickening of the vaginal mucosa. The epithelium becomes keratinized and eventually exfoliates large numbers of degenerated superficial cells during

estrus, with peak estrogen concentration before and during the preovulatory surge of lutenizing hormone (LH). This process is called cornification and desquamation of the vaginal epithelium (Robson, 1947). Although there are many studies of vaginal cytology during the different phases of the estrous cycle and pathological conditions of female reproductive tract are reported in different species (Sharma, 2016), there is a little information in buffaloes.

The classifications of vaginal epithelial cells in buffaloes described in this article are related to cells that have been reported in other species including: dog, cat (Burton, 2018; Allison *et al*., 2008), cow (Siregar *et al*., 20016), goat (Ola *et al*., 2006) and mouse (Harold, 2005). There are three main cell types of vaginal epithelium seen in the smears. They include parabasal, intermediate and superficial cells. The cells are classified according to the size of the nucleus relative to the size of the cytoplasm, and their shapes, which are described beginning with the most immature layer near the basement membrane and progressing superficially to the most mature layer.

Parabasal cells

Parabasal cells are small round cells with round vesicular nuclei and a small amount of cytoplasm (Figure 2A). Large numbers of these cells may be detected in vaginal smears swabbed from the prepuberty animals. (Feldman and Nelson, 2004; Amrulah *et al*., 2017).

Intermediate cells

Intermediate cells have variations in shapes and their sizes are about twice to triple the size of parabasal cells, depending on the amount of cytoplasm. They still have vesicular nuclei. In order to provide a clearer illustration of the transition of the epithelial cells during estrus cycle, they are subdivided into 2 stages include small and large intermediate cells (Figure 2B to D). As they increase in size their cytoplasm becomes irregular and angular. A study by Duran *et al*. (2015) using vaginal cytology as a tool for estrus detection in bubaline breeds in the Philippines also divided the intermediate cells into early and late intermediate cells, corresponding to small and large intermediate cells, respectively. However, some researchers or practitioners are not classifying them into these sub-groups (Siregar *et al*., 2016). It has been documented that only parabasal and intermediate cells have appeared in the vaginal cytology of cattle that have not entered puberty (Amrullah *et al*., 2017).

Superficial cells

Superficial cells are the largest vaginal epithelial cells. They have a polygonal and flat shape. The nuclei of these cells become pyknotic and then disappear as the cells undergo degeneration. Their cytoplasm is abundant, angular, and folded (Figure 2E to F). Superficial cells are commonly called cornified cells.

In addition, basal cells**,** from which all epithelial cells originate, may be detected in the vaginal smears. They are the smallest cells with a high nuclear-to-cytopalsmic ratio, and are rarely observed (Allison *et al*., 2008).

Other normal cytologic findings

Neutrophils (Figure 2D) have also been detected in the smears during the estrus cycle (Chaikhun *et al*., 2008).

Estrus cycle stage detection

The proportion of vaginal epithelial cells permits the classification of the estrus cycle stages.

The examination of vaginal cells has been used for determining the stage of estrus and the optimal time for mating in species such as dog, cat, mouse and rabbit. It is commonly used to determine the estrus cycle of dogs since the changes in this species are pretty consistent during the estrus cycle (Sharma, 2016). In cattle, however, these changes are not consistent (Rao *et al*., 1979).

The vaginal smears in proestrus are characterized by a mixture of parabasal, small and large intermediates, and superficial (cornified) cells along with variable numbers of neutrophils. It has been reported that a high percentage of neutrophils (30.6%) were detected in late estrus vaginal smears of swamp buffaloes (Chaikhun *et al*., 2008). This may correlate with the high estrogen concentrations at estrus that cause neutrophilia (Azawi, 2008).

As proestrus continues, there are progressively higher percentages of large intermediate and superficial cells present with fewer parabasal and small intermediate cells. After serum estrogen peak in estrus the main population of cells presented in the smears are superficial cells (Duran *et al*., 2015; Allison *et al*. 2008).

Kurude *et al*. (1993); Mingoas *et al*. (2009) reported that cornified cells formed about 50% and 47% of the cells in the smears of normal cycling cows during estrus, respectively. This corresponds to a study by Siregar *et al*. (2016) which investigated vaginal cells during the estrus cycle in synchronized Aceh cattle. They found the highest proportion of cornified cells (46.09%) in estrus. In addition, the highest numbers of cornified cells (79.6%) were detected in smears from the first estrus cycle from vaginal vestibules and vaginas of synchronized Holstein and crossbred cows in estrus (Macák *et al*., 2011). In buffaloes, a predominance of cornified cells were observed in the smears of three bubaline breeds (Native Philippine Carabow, Bulgarian Murrah and Brazillian Murrah) during estrus with ranges of 46% to 66%, 39% to 64% and 55% to 67%, respectively. A conception rate of 100% after artificial insemination was observed in each of the breeds inseminated with these cornification indexes with visible estrus sympoms (Duran *et al*., 2015). Although the population of cornified cells is dominant in this phase, it is still relatively low in contrast to the 90% proportion of cornified cells that have been found in dogs in estrus (Allison *et al*., 2008). The percentages of cornified cells in other species such as sheep (Zohara *et al*., 2014) and goats (Leigh *et al*., 2010) have been reported to range from 60 to 90% on the day of estrus. These variations could be due to genetic factors in each specie as well as environmental conditions (Mingoas *et al*., 2009).

The cornification index is beneficial for determining the progression of estrus stages since the cornification and desquamation of vaginal epithelium is due to estrogen's activity (Sharma, 2016). Highest cornification index expresses as the percentage of cornified cells detected in the smears of estrus period that exit highest serum estrogen during cycle (Siregar *et al*., 2016). In the cycling buffalo, the reproductive tract is usually under progesterone influences 14 to 15 days of its 21 days cycle. It is under significant estrogen influence for only about 1 day (immediately preceding standing heat) (El-Wishy, 2007). This short period of high estrogen may simplify to estimate the perfect timing for insemination via the high cornification index detection. It can be especially helpful to indicate the optimal time for insemination in cases of silent heat, which are common in buffaloes (Duran *et al*., 2015). Their short period of high estrogen concentration may be related to the lower percentage of cornified cells detected in buffalo vaginal smears in estrus when compared with those

of the other species, such as dogs.

High percentage of parabasal cells is a characteristic of diestrus (Duran *et al*., 2015; Kurude *et al*., 1993). An increased number neutrophils and intermediate cells were also reported (Subramanian and Pattabiraman, 1988). Increases of neutrophil during diestrus is related to the data reported by Ayen *et al*. (2012) which determined the defense cells profile of cervical mucous during follicular and luteal phases in river buffalo. Parabasal and intermediate cells were dominant when progesterone became dominant in diestrus (Siregar *et al*., 2016).

There are many estrus prediction parameters. In order to characterize stages of the estrus cycle more accurately, vaginal cytology should be determined in correlation with other techniques; observation of visual signs of the female external genitalia, behavior of cows and bulls, gyneco-clinical parameters, biochemical parameters (such as vaginal mucous fern pattern and cytology), serum estrogen and progesterone profiles, or ultrasonography analysis. A combination of three parameters provides an estrus detection efficiency rate of more than 70%, and detection efficiency rates rise as the number of combined parameters increases. (Selvam and Archunan, 2017; Siregar *et al*., 2016).

Pregnancy

There is no data on vaginal cytology in pregnant buffaloes at present. In cows, it is well known that intermediate cells increasingly predominate, followed by parabasal cells, but white blood cells are absent during most stages of pregnancy. This could serve as an indicator in the confirmation of pregnancy (Hussain and Khan, 1979). The predominant number of intermediate cells together with parabasal cells is similar in diestrus which involves serum progesterone influence.

Vaginal cytology of swamp buffalo at 6 months of pregnancy was observed from our field practice in Thailand (Figure 3). The vaginal epithelia cells were present in large numbers. The main population of the cell smears were intermediate cells which were composed of small and large cell stages. Some parabasal and superficial cells were detected but white blood cells were not present. These finding reflect the same trend as mentioned above in cows. In addition, foam cells were occasionally observed in the smear. These cells are small intermediate cells or parabasal cells containing numerous clear cytoplasmic vacuoles (Figure 3D). In dogs, they are seen on smears during diestrus and anestrus (Olson *et al*, 1984). Their significance is not known.

In cows, an increase in the number of neutrophils is observed during 265 to 275 days of gestation and 2 to 3 days before parturition, which could be due to the increase in vascularity associated with the influence of estrogen rising in these periods. An increase in parabasal cells can be detected after parturition while intermediate cells decrease due to changes in the estrogen and progesterone ratio following parturition (Sharma, 2016). Investigation into this issue in buffalo should be carried out in order to get more information, including research on other stages of gestation and after parturition.

Pathological conditions

Vaginal cytology can be used in bovines in diagnosing reproductive disorders such as hypofunction, persistent corpus luteum, cystic ovary and metritis (Gospodinov *et al*., 1987). These pathological conditions may result in increased calving intervals and decreased conception rates

which can effect animal production and cause economic losses for buffalo farmers.

Subramanian and Pattabiraman (1988) observed cornified and partly cornified superficial intermediate cells in bovine cystic ovaries. In addition, our observation revealed intermediate cells that vary in size in the vaginal smear of a swamp buffalo diagnosed with a follicular cyst as shown in Figure 4. Large numbers of neutrophils, some lymphocytes and yeast colonies were detected which indicated yeast vaginitis. The vaginal epithelial cell types detected in the smear of any ovarian cyst condition may reflect the animal's estrogen and progesterone levels. Nowadays, together with the progesterone profile, ultrasonography and echotexture analysis provide diagnostic tools for ovarian cysts in buffaloes with the wall thickness being used for the differentiation between follicular and luteal cysts (Noseir and Sosa, 2015).

Vaginal smears obtained from animals with inflammation of the vagina or uteri are characterized by large numbers of white blood cells such as neutrophils together with the vagina epithelial or endometrial cells, respectively. Vaginitis may be caused by irritation in the urogenital passage, bacteria, yeast or other organism infections. Krishna and Dharani (2010) reported that severe vaginitis occurred in near full term Murrah buffaloes. The perineal relaxation due to the changes in hormonal profiles near, and during full term caused vaginal exposure to the environment and thus attracted infections.

Moderate to large numbers of neutrophils characterize acute vaginitis. In addition, neutrophils, lymphocytes and macrophages may be seen in more chronic conditions (Allison *et al*., 2008). If a bacterial infection is the cause, neutrophils often show degenerative changes (swollen, pale nuclei with a loss of segmentation) and may contain phagocytized bacteria. The proportion of vaginal epithelial cell types detected in the smears should be related to the estrogen and progesterone levels in blood circulation (Sharma, 2016). Vaginitis can be associated with the presence of epithelial cells displaying atypical cellular features in response to the inflammatory process.

Metritis and emdometritis are most commonly encountered under field or farm condition in buffaloes (Azawi, 2006). The incidence of uterine infection in buffaloes is much higher than in cattle (Sheldon *et al*., 2002; Hanafi *et al*., 2008). In cattle, increased numbers of neutrophils and endometrial cells in vaginal smears has been shown to be associated with metritis while not much difference in vaginal epithelial cells is observed during the 3 weeks after parturition compared to the normal condition (Sharma, 2016). However, up to this date, no data has been reported on buffalo.

Although, vaginal cytology may give some information about metritis in cattle, ultrasonography and endometrial cytology are useful and relevant for diagnosing endometritis, especially in the subclinical condition during which cows are clinically healthy and show no evidence of mucopululent vaginal discharge (Barlund *et al*., 2008; Salah and Yimer, 2017). These diagnostic methods may apply to buffaloes as well.

Vaginal cytology of repeat breeders and anestrus cows has been reported (Kurude *et al*., 1993). For instance, an increased number of white blood cells and intermediate cells were observed in the repeat breeders while the number of cornified cells decreased. However, no data on buffalo has been documented.

POTENTIAL FOR FURTHER STUDY

Buffaloes are poor breeders. Silent heat is one of the major difficulties in understanding reproductive parameters and assisted reproduction in buffaloes (*Mondal et al*., 2008). Good estrus detection can determine the optimum time for insemination. It is the key to success in both natural mating and AI program management. There are many estrus detection methods that have been reported in buffalo. Acceptance of the male is considered as the most reliable estrus indicator in buffaloes, but the detection efficiency varies a lot (Van Eerdenburg *et al*., 2002; Suthar and Dhami, 2010). Vaginal cytology is a simple and non-invasive technique that can be used for the determination of the buffalo estrus cycle and AI timing (Chaikhun *et al*., 2008; Duran *et al*., 2015). However, few studies have been reported in buffalo. Thus, more vaginal cytology investigations in both natural and hormonally induced estrus cycle buffaloes should be performed in order to get more understanding of their physiology. It would be a cost-effective model for farmers. In addition, observation should be carried out in both seasonal and non-seasonal breeding periods in order to get complete data throughout the year which may provide valuable information. The combination of vaginal cytology and other estrus detection methods can improve the efficiency of estrus detection and increase the rate of buffalo reproduction.

Vaginal cytology may assist in determining pregnancy status, estimated time of parturition, and pathological conditions of the reproductive tract such as vaginitis, metritis or neoplasm. Further study should provide useful information for their application to field practice. The technique has been established as a good clinical indicator in small ruminants (Ola *et al*., 2006), but there is little data regarding its use in buffaloes.

Alternatively, staining techniques used in the field or by rural farmers may be adapted by using natural dye extract from the plants that grow in each region (Suebkhumpet and Sotthibandhu, 2012). The use of natural dyes from plants has gained interested since most of them contain plenty of dye and they are eco-friendly materials.

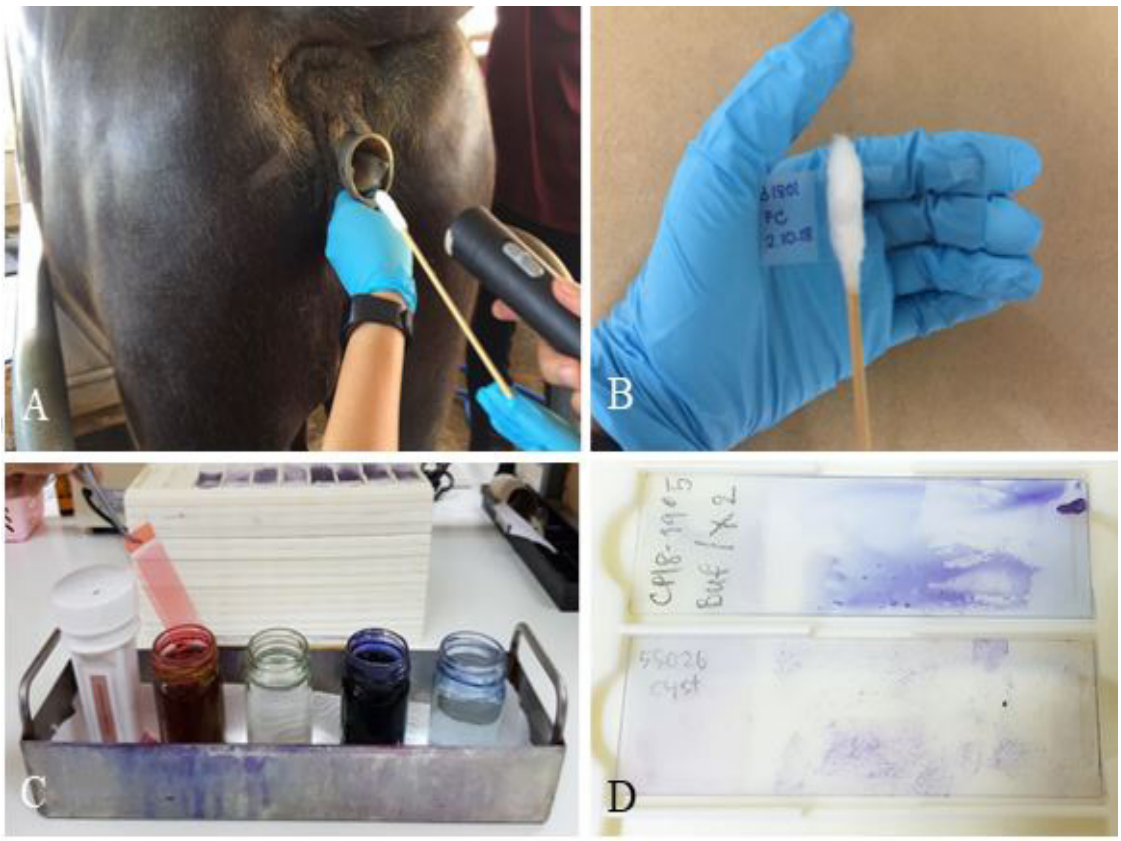

Figure 1. Preparing the vaginal smears for cytological study.

A: A cotton swab is inserted into the vaginal opening compatible with vaginal speculum.

B: A cotton swab is rolled onto a glass slide.

C: Staining solution: Diff-Quick stain.

D: The stain smears are ready for examination under light microscope.

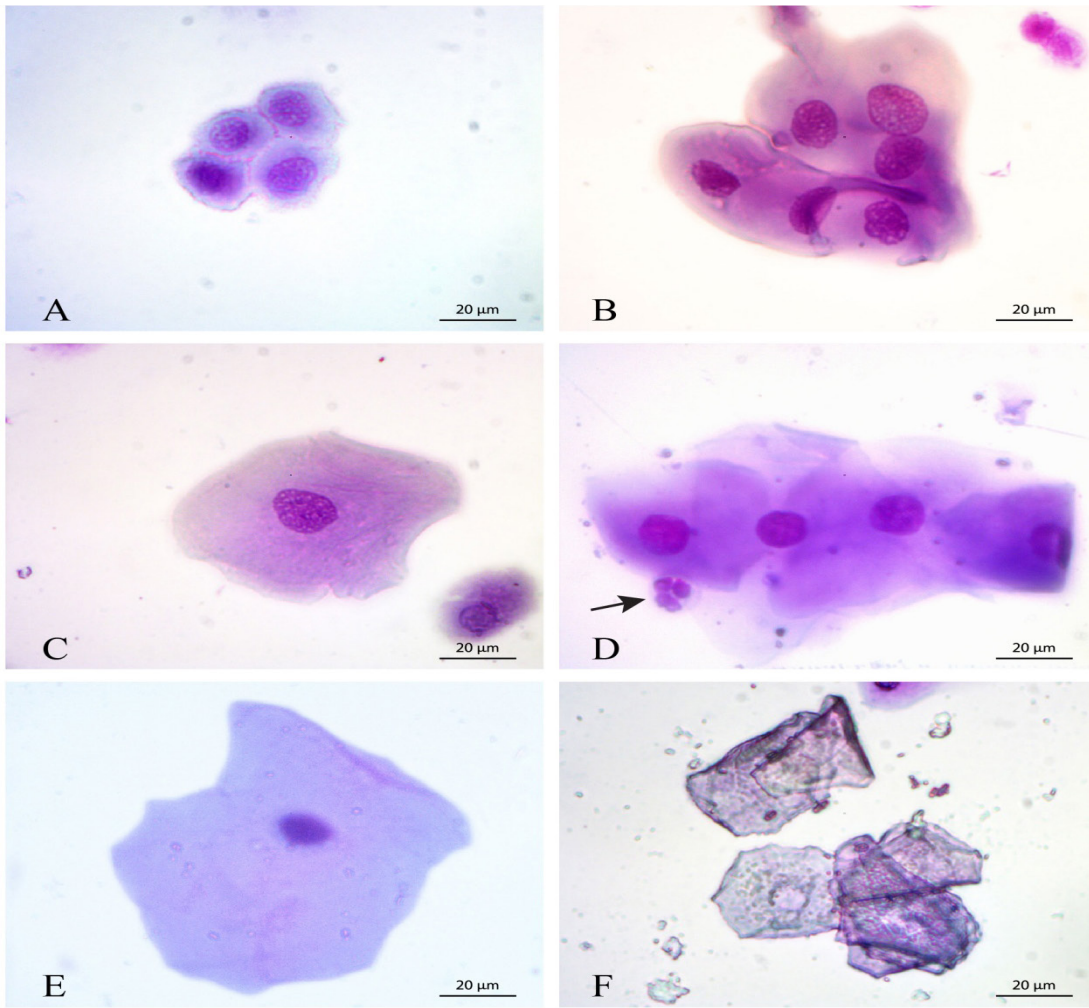

Figure 2. Epithelial cells from the vaginal smears of swamp buffaloes.

A: Cluster of parabasal cells.

B: Custer of small intermediate cells.

C: A large intermediate cell.

D: Neutrophil (arrow) and large intermediate cells.

E: A superficial cell with pyknotic nucleus.

F: Anucleated superficial cells and some superficial cells with small pyknotic nuclei and angular to folded borders. (Diff-Quick stain; 40X objective.)

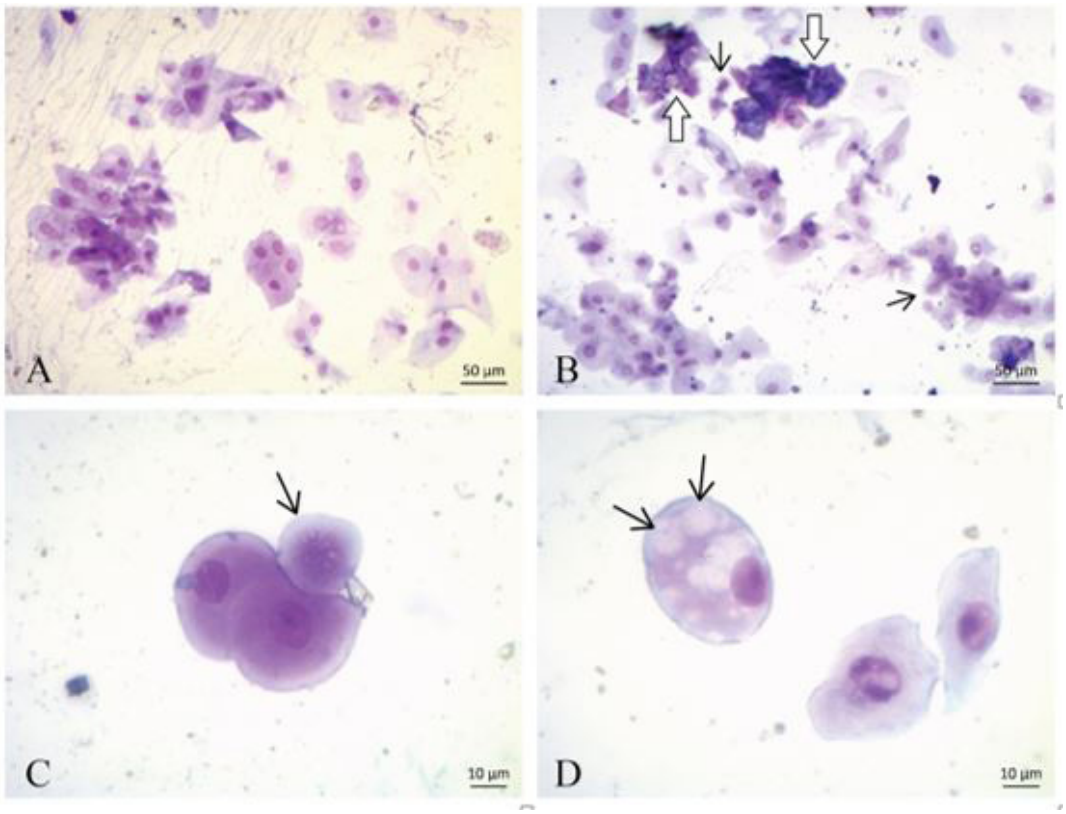

Figure 3. Vaginal cytology of swamp buffalo in 6 months of pregnancy.

A: Intermediate cells are dominant and arranged individually or in small clumps.

B: Groups of superficial cell are located (open arrows) among large number of intermediate cells. Some of parabasal cells are also detected in the smear (arrows).

C: A parabasal cell (arrow) and two small intermediate cells.

D: Foam cell with multiple cytoplasmic vacuoles (arrows). (Diff-Quick stain; 10X objective for panel A-B and 40X objective for panel C-D).

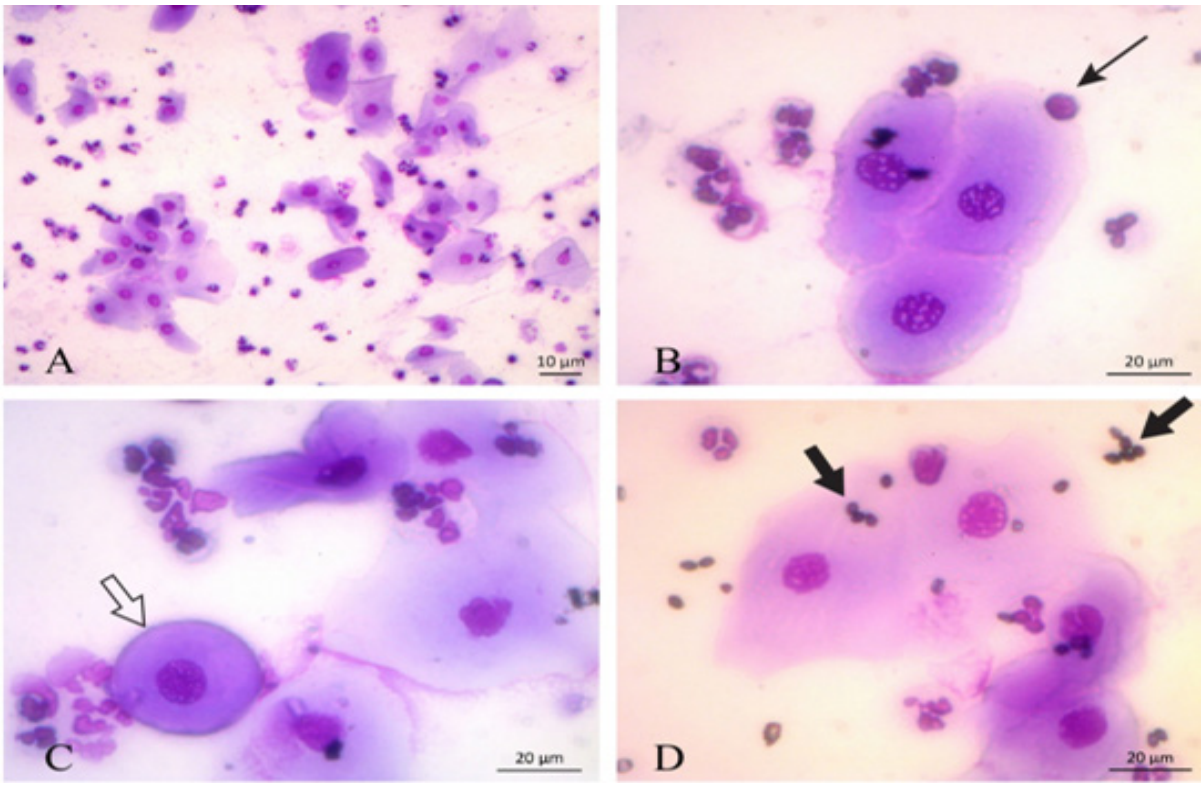

Figure 4. Vaginal smear, ovarian (follicular) cyst, swamp buffalo.

A: There are intermediate cells vary in size dominate, accompanied by many neutrophils in the smear (10X objective).

B: Intermediate cells are arranged in small clumps. Neutrophils and lymphocyte (arrow) appear.

C: Small intermediate cell (open arrow) is nearly round shape and contains smaller amount of cytoplasm than large intermediate cell.

D: Intermediate cells are presented with admixed neutrophils and yeast colonies (solid arrows). (Diff-Quick stain; 10X objective for panel A and 40X objective for panel B-D).

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