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Exfoliative cytology of the uterus and vagina during the follicular and luteal phases in one-humped camel (*Camelus dromedarius*)

YB Majama[1]*, GD Mshelia[2], Y Ahmed[1], Y Abba[3] & HD Kwari[1]

[1.] *Department Veterinary Anatomy, University of Maiduguri, PMB 1096, Maiduguri, Borno State, Nigeria*
[2.] *Department of Theriogenology, University of Maiduguri, Borno State, Nigeria*
[3.] *Department of Veterinary Pathology, University of Maiduguri, Borno State, Nigeria*

*Correspondence: Tel.: +2348038331556; E-mail: ymajama@yahoo.com

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Abstract

The exfoliative cytology of the uterus and vagina area was evaluated in one-humped camel during the follicular and luteal phases. This was carried out using intact genitalia collected from camels immediately after slaughter. A total of 86 genitalia and blood samples were collected and processed. The mean serum progesterone (P4) profile was determined and used to categorize the animals into the follicular (n=51) and luteal phases (n=35). The result of the differential cell counts of the exfoliated vaginal epithelial cells showed mean basal, superficial cells and leucocytes of 27.0%, 25.4% and 14%, respectively being the most prominent during the follicular phase, while the uterine smears were characterized by mean basal (25.6%) , intermediate cells (24.6%) and leucocytes (9.6%) respectively. During the luteal phase, the vaginal smears were characterized majorly by the intermediate (27.4%), parabasal cells (26.6%) and leucocytes (11.6%, while the uterine smears were predominantly characterized by intermediate (22.8%), superficial cells (22.6%) and leucocytes (17.8%) respectively. The result showed that the exfoliative cytology of the uterus and vagina are similar during both the follicular and luteal phases in camels, and so cannot be used alone to characterize these reproductive period. However, this finding has a significant potential application in the use of vaginal cytology alone to assess uterine cellular changes without necessarily invading the uterus under field conditions in camel.

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Introduction

The female camel reaches her full reproductive capacity at about 6 years of age (Khanvilkar *et al*., 2009), but can be bred at 2-5 years (Marai *et al*., 2009) up till 30 years of age (Mehari *et al*., 2013). This animal species has a unique estrous cycle compared to those of the ungulates. The phases of the cycle usually described for species with spontaneous ovulation (estrus and diestrus) do not exists in Camelidae unless the female is bred and has ovulated. In the absence of mating, there is only a succession of follicular waves with high variable rhythm (Tibary & Anouassi, 1997). Al-Eknah *et al*. (1993) showed four distinct uterine activity phases namely: the high, declining, low and increasing phases during the camelids estrous cycle. Because the camels are induced ovulators (Marie & Anouassi, 1986) with ovulation occurring after mating (Ayoub *et al*., 2003; Skidmore *et al*., 2005;

Ghazi, 2007). They have a very slow rise in peripheral serum progesterone concentration after ovulation and a short luteal phase of only 9-10 days in the non-pregnant animal (Skidmore *et al.,* 1995). The serum progesterone level depends on the reproductive state and age of the camel cow (Kamoun & Jemmali, 2014). In the empty postpartum camel, the serum progesterone concentration varies between 0 ng/ml and 0.41ng/ml with mean variations in the order of 0 to 0.38±0.04 ng/ml (Kamoun & Jemmali, 2014).

Cytological methods along with progesterone profiling have been used in characterizing the reproductive stages in some animal species (Abou-El-Roos & Abdel-Gaffer 2000; Doi *et al*., 2000; Mshelia *et al*., 2001; Ahmadi *et al*., 2005; Knauf *et al*., 2009). The applications of some reproductive biotechniques such as superovulation and embryo transfer in camels were based on a sound understanding of the reproductive biology, including an in-depth appreciation of the normal anatomy and histology of the reproductive tract of these species during different reproductive stages (Skidmore *et al*., 1998). However, there seem to be a paucity of information on the cytological changes in the uterus and vagina of the camels during the different reproductive stages. The aim of this study was to elucidate on the exfoliative cytology of the uterus and vagina during the follicular and luteal phases in camels.

Materials and Methods

Study area

This study was carried out in Maiduguri, Borno state, located in the North eastern part of Nigeria. The city is cosmopolitan in nature, situated at an elevation of 354 meters above sea level; and located between latitudes 11^{o} and 14^{o} N and longitudes 10 ^{o} and 14 ^{o} E within the conventional Sahel zone. Maiduguri has a total landmass of 50,778 square kilometers (BMLS 2007) and a population density of 1,738 people per square kilometer (NPC, 2006). Temperature ranges from 35-40 ^{o} C for most parts of the year, with two distinct seasons, the rainy season with a mean annual rainfall of 647mm (from July to October) and a prolonged dry season for the rest of the year (LCRI, 2007).

Animals and sample collection

A total of eighty six blood samples and intact genitalia were collected from one-humped female camels brought to the Maiduguri metropolitan abattoir for slaughter. The blood was collected before slaughter through jugular venopuncture and transferred into clean sterile vacutainer tubes. The genitalia were collected immediately after slaughter and placed on ice into clean polythene bags, and transported immediately to the Department of Veterinary Anatomy Research Laboratory, Faculty of Veterinary Medicine, University of Maiduguri for analysis. Serum was harvested by centrifugation at 2000-3000 rpm for 10 minutes and stored at -80 ^{o} C until used for progesterone assay.

Progesterone (P4) assay

The Camel Progesterone (PROG) ELISA Kit (My Bio Science, USA) was used for the P4 assay. This assay was based on the Biotin double antibody sandwich technology, and was carried out according to the manufacturer's instruction. Briefly the wells were pre-coated with progesterone (PROG) monoclonal antibody. A serial dilution of the standard solution (240 ng/ml, included in the kit) was made in test tubes as follows: 120 ng/ml, 60 ng/ml, 30 ng/ml, 15 ng/ml, and 7.5 ng/ml. The standard solution and blank were then arranged in the wells, with the first two wells being used for the blank sample.

The wells were coated with monoclonal antibodies .Thereafter; 50 µl of the standard and 50 µl of streptavidin were added to the respective standard wells. A total of 40 µl of the test sample was added to the remaining wells respectively, followed by 10 µl of the PROG antibodies labeled with biotin and 50 µl of streptavidin. These were then shaken gently, covered with the seal plate membrane and incubated at 37^{o} C for 60 mins. The seal membrane was then removed, drained and shaken to remove the remaining liquid and then washed with washing solution repeatedly for 5 times. Each well was then filled with the prepared washing solution and allowed to stand for 30 seconds. The liquid was then drained and 50 µl of chromogen A was added to all the wells including the standard well. Thereafter, 50µl of chromogen B was added and gently mixed up, and incubated for 10 minutes at 37^{o} C away from light for color development.

Exfoliative cytology

The recovered genitalia were incised using sterile scissors and forceps to expose the vaginal epithelia and uterine endothelia. Vaginal and uterine smears were obtained from each camel specimen using sterile swab sticks. The smears were obtained by inserting the swab sticks gently through the genital incisions made and rolled severally over the epithelial and endothelial linings of the vagina and uterus respectively, until soaked. The soaked swab

sticks were then withdrawn, smeared onto clean glass slides, air dried and fixed with methanol, stained with Giemsa stain and examined under light microscope and interpreted according to Mshelia *et al. (*2001).

Statistical analysis

Concentrations of progesterone (P4) obtained were summarized into mean ± SEM and analyzed using Students' T-test. Differences between the proportions of exfoliated cells in the uterus and vagina were analyzed using Z-Test. All the analyses were carried out using statistical software, Graphpad prism (version 6) and P-value was set at $p<0.05$.

Results

Serum progesterone (P4) assay

The result of the serum P4 assay was presented in Table 1. The serum P4 profiles for all the camels examined ranged from 0.6 to 4.6ng/ml. Those animals with P4 values <1ng/ml (n=51, mean of 0.89 ± 0.16ng/ml) and those with values >1ng/ml (n=35, mean of 1.61 ± 0.81ng/ml) were considered to be in their follicular and luteal phases respectively and so were categorized accordingly. (Kamoun & Jemmali., 2014, Skidmore *et al*., 1996)

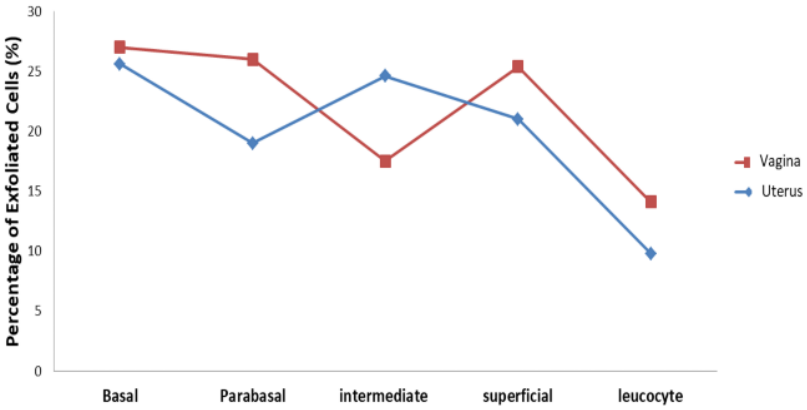

Figure 1: Differential cell counts of exfoliated cells of the uterus and vagina during the follicular phase in one-humped camel (*Camelus dromedarius*)

Exfoliative cytology

The result of the exfoliative cytology of the uterus and vagina was presented in Figure 1 and 2. The result showed that during the follicular phase, the uterine smears were characterized by the presence of basal (25.6%) and intermediate (24.6%) cells. Similarly, the vaginal smears were also shown to be predominantly characterized by the presence of basal (27.0%) and superficial (25.4%) cells; with marked presence of leucocytes in both the uterine and vaginal smears during this reproductive phase. During the luteal phase, the differential cell counts of the uterine smears showed predominant presence of intermediate (22.8%) and superficial (22.6%) cells. The result also showed that the vaginal smears were characterized predominantly by the presence of intermediate (27.4%) and parabasal (26.6%) cells. The presence of leucocytes was also observed in both the uterine and vaginal smears. The representative smears of the uterus and vagina during the follicular and luteal phase were shown in Plates I and II, respectively.

Discussion

Out of the total number of camel serum samples assayed for progesterone in the present study, 59.3% of them were found (based on their P4 profiles) to be in the follicular phase; while 40.7% were in the luteal phase. The mean serum P4 value observed for camels in the follicular and luteal phases in the present study were 0.89± 0.16ng/ml and 1.61 ± 0.81ng/ml respectively. This finding is consistent with previous reports in the camel (Skidmore *et al*., 1996; Basiouni, 2007; Ali *et al*., 2011). Generally, serum P4 value is expected to be low (< 1ng/ml) in the non-pregnant camels (Agarwal *et al*., 1989; Tibary & Anouassi, 1997); and values of as low as 0.04 ±0.4 ng/ml (Cristofori *et al*., 1986) and as high as 0.98 ± 0.26 ng/ml (Ali *et al*., 2011) have previously

Table 1: Serum Progesterone (P4) levels during the follicular and luteal phases in one-humped camel (*Camelus dromedarius*) in Maiduguri, Nigeria

Reproductive Phases	Number of animals	P4 Conc. (ng/ml)	
		Mean ± SEM	Range
Follicular phase	**51**	0.89^{a}±0.02	0.6-1.0
Luteal phase	**35**	1.61^{b}±0.14	1.1-4.6

^{a,b} means with different superscripts are significantly different at $p<0.05$

been reported in camelids. Camels are known to be induced ovulators (Ayoub *et al*., 2003), usually ovulating after mating (El-Wishy & Homeida, 1984; Skidmore, 2005; Basiouni, 2007; Marai *et al*., 2009; Kamoun & Jemmali, 2014). In the mated animal, corpus luteum forms 3-4 days after ovulation and grows from a diameter of 0.7±0.2 cm from day 3 post mating to 2.2 ±0.1 cm by day 9. Such CLs have been shown to have a secretory life span of 8.5 ± 0.5 days with serum P4 concentration ranging from ≤ 0.5ng/ml in the first 3-4 days post

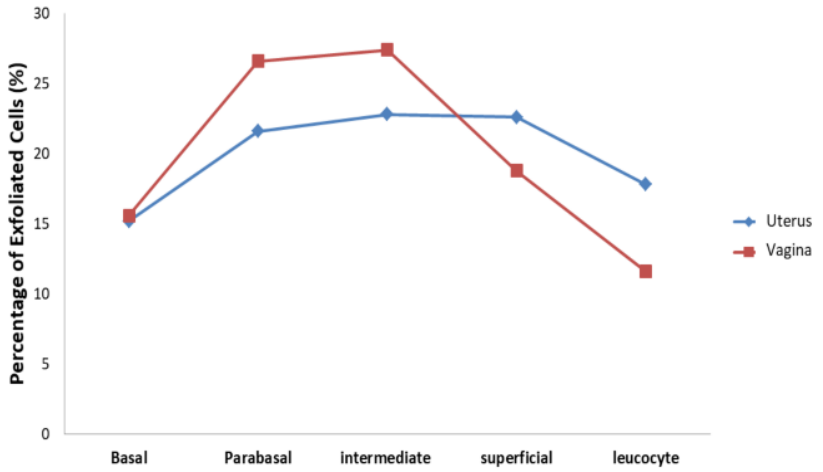

Figure 2: Differential cell counts of exfoliated cells of the uterus and vagina during the luteal phase in one-humped camel (*Camelus dromedarius*)

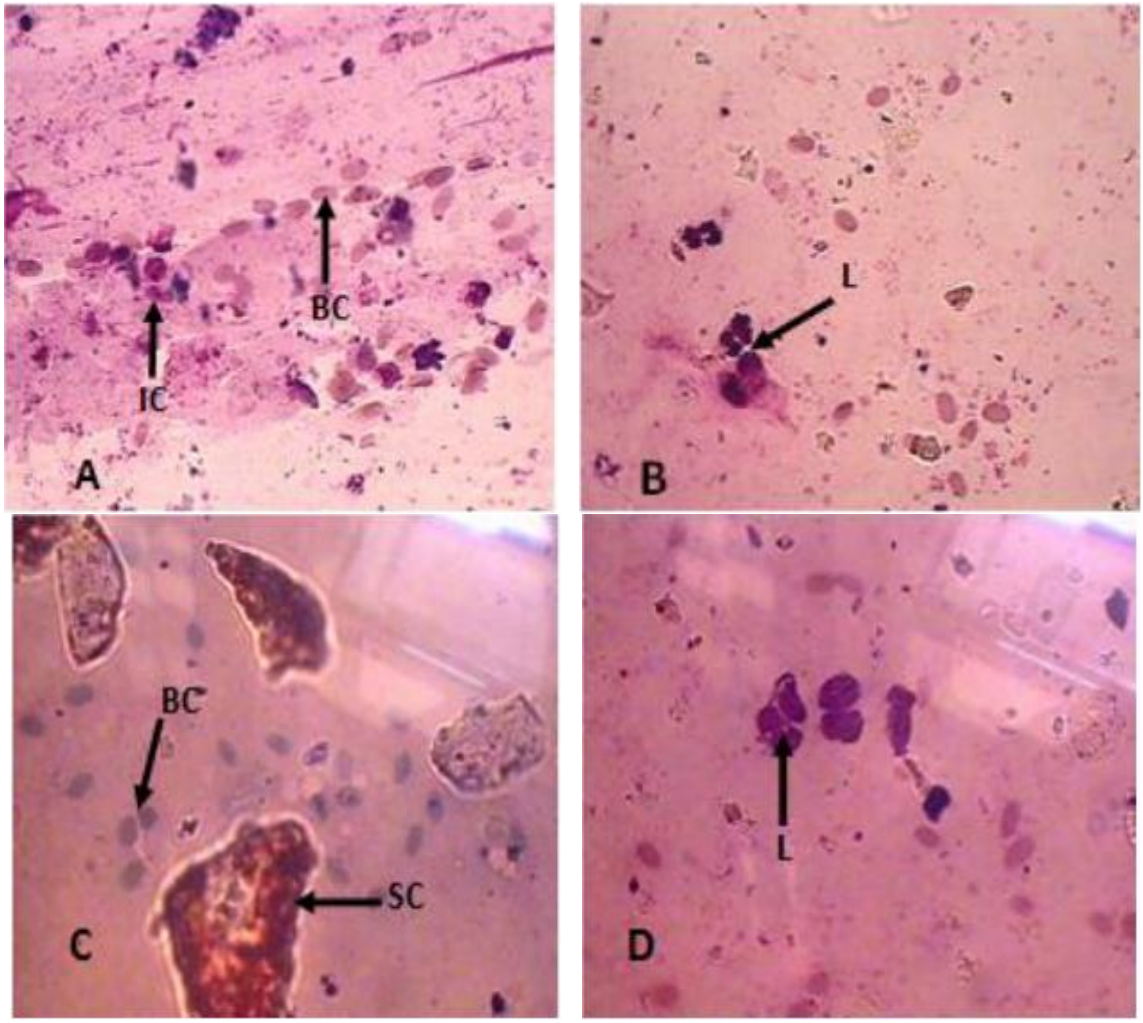

Plate I: Uterus and vaginal smears of one-humped camel during the follicular phase showing: (**A**), predominant Basal-BC and Intermediate-IC cells in uterine smears, (B), Leucocytes-L in uterine smears, (**C**), Basal-BC and Superficial-SC cells in vaginal smears and (D), Leucocytes -L in vaginal smears (**D**)

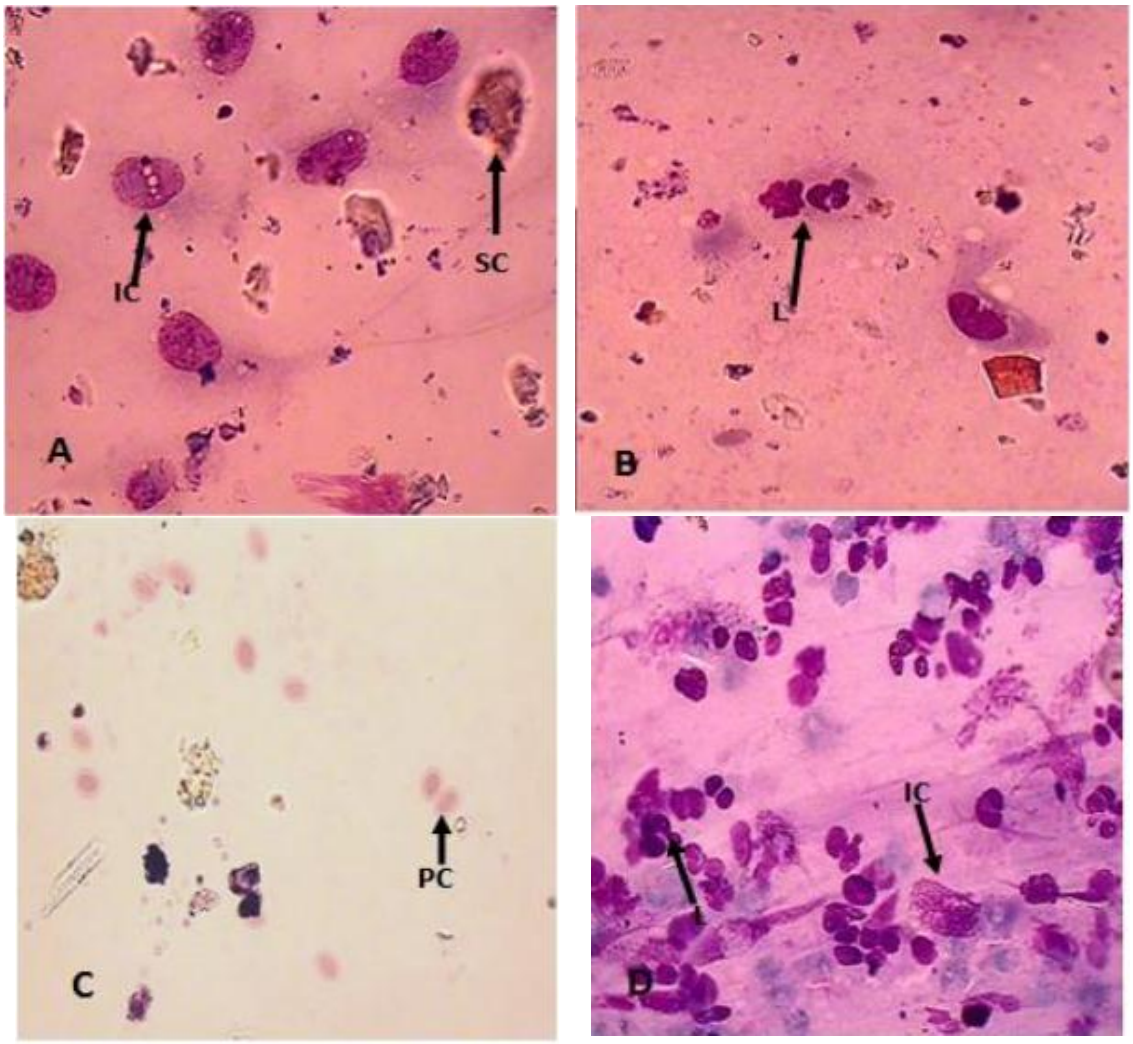

Plate II: Uterus and vaginal smears of one-humped camel during the luteal phase showing: (A), predominant Intermediate-(IC) and Superficial-(SC) cells in uterine smears; (B), Leucocytes-L in uterine smear; (C), Parabasal-PC cells in vaginal smears; and (D), Intermediate (IC) cells and Leucocytes-L in vaginal smears

ovulation to peak levels of 2.6ng/ml by day 8 when the CL begins to regress (Skidmore, 2005). Skidmore *et al*. (1996) showed that P4 levels begin to decrease sharply from days 9-10 to reach baseline values of 0.5ng/ml from days 10-12 in the absence of pregnancy. Since the primary source of P4 in the female camelids is the corpus luteum, this goes to show why serum P4 levels remains low (< 1ng/ml) during the follicular phase in the non-pregnant camels (Skidmore *et al*., 1996; Ayoub *et al*., 2003). However, in successful mating with resultant pregnancy, the serum P4 value remains high (>1ng/ml) throughout gestation (Kamoun & Jemmali, 2014) reaching peak values of up to 6.45±0.65ng/ml about 3 days before parturition and drops to somewhere in the border of 2.47ng/ml at parturition (Ayoub *et al.*, 2003).

The measurement of the serum hormonal profiles would offer better understanding of the reproductive stages in animals (Abou-El-Roos and Abdel-Ghaffer, 2000; Doi *et al*., 2000; Kamoun & Jemmali, 2014), and majorly so in the pregnant camelids (Kamoun & Jemmali, 2014). Although progesterone and estradiol profiles have been described during different reproductive stages in many domestic animals, only very few reports are available in the camelids (Ayoub *et al*., 2003). In the present study, we hypothesized that serum P4 values can be used to broadly categorize the follicular and luteal phases in the non-pregnant camels. All the blood samples analyzed in the present study, none of the contributing animals had noticeable corpus luteum on their ovaries. Abdel-Rahim (1989), reported that the presence of P4 is

necessary for the manifestation of estrus and sexual receptivity in camels. In non-mated camels, the mature follicle becomes atretic and gives way for new follicular wave that will initiate estrus and sexual receptivity (Skidmore *et al*., 1996). It was also been reported that the source of P4 in non-cycling camels could be of ovarian or adrenal origin (Ayoub *et al*., 2003). Earlier on, Marie & Anouassi (1987) observed that ovulation could occur in non-mated camels exposed to the sight, sound and smell of a male after a prolonged absence, thus suggesting the possibility of spontaneous ovulation in camel (Ayoub *et al*., 2003). If this is the case, the resultant CL from such ovulation is short lived similar to what could be observed in sterile matings (Elias, 1990). One of the major limitations of this study is the lack of information regarding the reproductive histories of these camels that could have allowed for the correlations of the findings in the present study with specific reproductive status of the animals. That would have been very interesting, however, the serum P4 values observed in the present study is significant as it has elucidated on the importance of P4 profiling in determining the reproductive status of the Camels under natural field conditions.

Data in the present study demonstrate that during the follicular phase, the uterine smears were characterized predominantly by the presence of basal cells and intermediate cells, while during the luteal phase, the presence of intermediate and superficial cells were predominantly observed. Similarly, the study also showed that basal and superficial cells were the most predominant exfoliated cells in the vaginal smears during the follicular phase, while intermediate and parabasal cells were most predominant during the luteal phase. Although slight changes have been observed in the exfoliated vaginal cytology of the camels during the follicular and luteal phases in this study, the distinct pattern previously observed in the bitch could not be sustained. This is likely due to the variation in the hormonal patterns during the estrous cycle in this specie. In previous studies in bitches (Olson *et al*., 1984; Mshelia *et al*., 2001), it was shown that superficial cells were predominantly high in vaginal smears during the transition from proestrus to estrus (or the follicular phase). This period of marked proliferation of superficial epithelial cells in vaginal smears in the bitch, coincide with the period of high stimulatory effect of secretory estrogens in the bitch (Mshelia *et al*., 2001).

In one study, Knauf *et al*. (2009) showed that basal, parabasal and intermediate cells were the most predominant exfoliated cells in vaginal smears of non-human primates during the proliferation (follicular) phase; with the superficial and intermediate; and parabasal cells predominantly being observed during the ovulatory (estrus) and secretory (luteal) phases respectively. In the mare, vaginal smears have not been able to clearly distinguish the different stages of the estrus cycle. In one report (Abou-El-Roos & Abdel-Gaffer, 2000), a relative increase in superficial cells and leukocyte infiltration has been attributed to decreased plasma concentration of P4. It was also observed in elephants, that the period characterized by marked presence of superficial cells in vaginal smears corresponded with the period of low serum progesterone concentration (i.e. follicular phase or proestrus and estrus), while marked presence of parabasal and intermediate cells were associated with the luteal phase (or metestrus and diestrus) when P4 values begin to increase (Doi *et al*., 2000). The report also showed that increased presence of leukocytes in the vaginal smear is related with the period of low serum P4 concentration.

The finding in the present study has shown that vaginal cytology cannot be adequately used alone in camels (as in the bitch) for the staging of their follicular and luteal phases. This is because of the similarity in pictures of the exfoliated cells of the uterus and vagina during these reproductive phases. Previous reports in dairy cow (Ahmadi *et al*., 2004) and camels (Ahmadi *et al*., 2005), have not shown any significant differences between cervical and uterine smears during different reproductive phases, suggesting that cervical smears could be used in evaluating uterine conditions. The similarity in the exfoliative cytology of the uterus and vagina could therefore be of advantage as it can allow the use of vaginal cytology alone for the assessment of uterine conditions without necessarily invading the uterus.

In conclusion, the present study has shown that serum P4 levels can be used to categorize camels into the follicular and luteal phases with mean values of 0.89 ± 0.16 ng/ml and 1.61 ± 0.81 ng/ml respectively. The exfoliative cytology of the uterus and vagina showed some similarities in the uterine and vaginal smears during both the follicular and luteal phases in the camels. The significance of this finding is that vaginal cytology can be used to reflect cellular changes in the uterus without necessarily invading this organ. This could be an important information in reproductive/breeding

synchronization as well as basic data in spontaneous ovulating breeds and species.

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