Advances in Biological Sciences Research, volume 14
Proceedings of the 3rd KOBI Congress, International and National Conferences (KOBICINC 2020)

Vaginal Smear and Neutrophil Count as an Alternative Method for Estrous Phase in Female Tiger (*Panthera tigris sumatrae*, Pocock, 1929)

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ABSTRACT

Detection of the estrous cycle is essential for basic reproductive aspects in all animals. Both natural mating and assisted reproductive techniques require estrous detection for breeding programs which will assist to maintain the tiger population in captivity. This research aims to explore alternative methods for tiger conservation in the remote area. Vaginal smear and neutrophil count were used to determine the phase of estrous cycle, and it easily applied on the field. Vaginal smears and blood smears were collected twice within interval 6 months n a female tiger at Tambling Wildlife Nature Conservation Rescue Centre. While tiger was restrained mechanically, blood samples were collected from the coccygeal vein and the vaginal smears were collected intravaginal. All procedure followed the animal welfare protocol. Vaginal smears were stained by 10% Giemsa. Basal/parabasal, intermediate, and superficial/cornified cells were identified to determine the reproductive periods under the microscope. Blood smears were stained by Giemsa-Wright. Neutrophils were counted within 200 white blood cells count under the microscope. The result showed that female tiger was in the follicular phase and neutrophil count was 78 cells at the 1st sample collection. The 2nd sample collection resulted in luteal phase with 55 neutrophils count. This research suggests vaginal smear and neutrophil count can be used as an alternative method of tiger estrous phase detection. In addition, these methods have economical and practical value.

Keywords: *vaginal smears, neutrophil count, tiger, estrous, semi ex-situ*

1. INTRODUCTION

Assisted reproductive technologies are being developed for wild cat conservation [1]. Artificial breeding technology and reproductive technology can contribute to species conservation [2]. Information of the basic reproductive aspects of wild mammals is scarce [3]. In vivo fertilization (IVF) successfully performed on tiger. However, effective and efficient basic research approach and applied reproductive biotechnology using mobile laboratory research are important for wildlife breeding programs [4]. This method widely used for companion animals [5], farm animals [6,7] and possible for wildlife animals [8,9,10]. The procedure can reliably be used for diagnosing phase of estrous and optimum time for mating [12].

Neutrophils often presence on vaginal smears observation [13,14]. Neutrophil infiltration into the vagina (female reproductive tract) is essential to maintaining the estrous cycle. Neutrophils maintain the estrous cycle through control of steroid hormone levels [15]. Both of immune response and proper structural integrity of the vagina require estrogen action in the vaginal epithelial cells [16]. Relationship between neutrophil blood count and neutrophil infiltration during the estrous cycle has been proven clinically by [17]. Neutrophils have crucial roles on female reproductive tract on the process of fertilization and immune response [18].

Tiger species is the second most researched species from Felidae family after the domestic cat [19]. Despite of those high frequency of researches, tiger's population is declining due to deforestation [20,21,22,23,24], poaching [25,26,27], and human-

tiger conflict [28,29]. Estrous detection on the tiger leads to the optimum mating in breeding programs. Maintaining tiger's population is a part of tiger conservation. This research aims to explore alternative methods to detect the estrous phase on tiger species. By combining vaginal cytology and neutrophil blood count on the field, this research expects to minimize the risk of damaging the sample during transportation to the laboratory.

2. MATERIALS AND METHODS

2.1. Subject

This research was conducted in Tambling Wildlife Nature Conservation Rescue Centre (Tambling). The adult female tiger was translocated to the rescue centre on 2009 from Jambi because of human-tiger conflict. Tiger was given *ad libitum* water and live prey (10% of body weight) twice a week. Tiger was conditioned in a solitary cage (6 m × 6 m) and routinely moved to habitual cage in every 2 weeks. Individual cages were partly covered with roof adjusting for tropical condition. Tiger was freely choosing its position in the individual cage. Both cages had natural light setting. Animal husbandry and veterinary care were designed to follow *animal welfare principle* at Tambling Rescue Centre. This research was approved by the Ethics and Research Committee at the Faculty of Medicine Universitas Indonesia (Protocol Number 20-01-0058) and Secretariat of Scientific Authority for Biodiversity Indonesian Institute of Sciences (LIPI).

2.2. Sample collection

Vaginal smears and blood samples were taken from a female tiger twice during the sampling period. Samples collections were done within interval 6 months engaging the medical check-up routine by the Tambling's veterinarians. The samples collection could not be conducted on daily basis to minimize human-animal interaction at Tambling Rescue Centre. Tiger was restrained mechanically on a squeeze cage without anaesthesia. All procedures were conducted with safety consideration between the animal and the operators. All samples were collected *lege artis* and aseptically under 3 minutes (Figure 1).

Blood samples were drawn through lateral coccygeal vein aseptically. Sterile syringe (3 ml) and needle (22G) were used to collect 0.5 ml blood from the tiger. The needle was removed from the syringe. Blood smears were made as soon as possible after the blood collection. Clean object glass was given a drop of fresh blood from the syringe. Another object glass was put about 30° to the drop of blood on the first object glass. The second object glass was pushed quickly and smoothly across the full-length after the blood spread within 2 – 3 mm of the first object glass [30,31]. The fixation of the blood smears was using methanol within 2 – 3 minutes. Blood smears were stained after dried over fixation [32].

(a) (b)

Figure 1. Sample collection on a female tiger with mechanical restraint. (a) cage designed (2 m x 2 m and 1,5 m height) for mechanical restraint. (b) safety protocol between the tiger and the operators during sample collection.

Vaginal smears were collected after the blood sampling. Sterile cotton swab slowly introduced about 4 cm into the vagina until reached the vaginal walls. Sterile cotton swab then gently rotated intravaginal and rolled onto a clean object glass [11]. Object glass was fixed by Bunsen lamp.

2.3. Vaginal smears and blood smears staining

Vaginal smears and blood smears staining were conducted in different area from the tiger's enclosure to minimize debris on the smears. The area for staining was conditioned to had a room temperature, avoid direct sunlight and rainy or windy weather. Vaginal smears were stained with 10% Giemsa for 15 minutes [10,33,34]. Blood smears staining were used combination of Giemsa-Wright solution. Blood smears were dipped into Wright solution for 2 minutes then rinsed with aquadest solution. Blood smears were dipped into 10% Giemsa solution for 15 minutes [35]. Vaginal smears and blood smears were rinsed by aquadest solution after staining.

2.4. Vaginal smears and blood smears analysis

All analysis of the smears under the microscope were conducted after the slides dried. Identification of the cells on vaginal smears was done through microscope at 40× and 200× magnification. Basal/parabasal, intermediate, and superficial/cornified cells were observed to determine the cycle of estrous cycle [10,33]. Type of cells on the smear were evaluated as traces (+/-); none (-), little (+), medium (++), or dense (+++) [13,14].

Neutrophil blood count was conducted through microscope at 40× and 400× magnification. Neutrophils were counted within 200 cells of white blood cells count (differential leucocyte) under the microscope [31,36,37]. Neutrophil blood count is higher on follicular/ovulatory phase than luteal phase. Lower neutrophils count on the blood are resulted from the neutrophil translocation to the female reproductive tract. Following [17] and [18] hypothesis, neutrophil blood counts were added to determine the cycle of oestrus cycle with vaginal smear method. Mobile phone camera that put to the ocular lens of the microscope were used to document all photos in this research.

3. RESULT AND DISCUSSION

3.1. Vaginal smears examination

Epithelial cells from the vagina were observed under the microscope using sterile cotton swab technique. The results from the present study showed basal/parabasal, intermediate, and superficial/cornified cells were observed on the vaginal smears. Considering this research was conducted on a female tiger as a wild animal without anaesthesia, rapid and practical sampling techniques were required. Cotton swab technique for vaginal smear was chosen for this research. Cotton swab technique is successfully applied to cheetah [38] and African lion [11]. Cell identifications from vaginal smears in this research showed similar result to the previous researches [11,14,39,40,41].

From the vaginal smear observations, first smears indicated the female tiger was on follicular phase at the oestrous cycle. Intermediate (++) and superficial/cornified cells (+++) were dominant on the vaginal smears (Figure 2). Second vaginal smears indicated luteal phase on the estrous cycle. Basal (+), parabasal (++), and intermediate cells (+++) (Figure 3) were dominant on the second vaginal smears collection. Type of cell densities of the vaginal smears on this research were suitable to previous studies [13,14]. The total population of the cells on the smears in domestic cat are changing at the 1st – 4th day of follicular phase according to other researchers [43,44]. Specifically, Cornified cells increase from

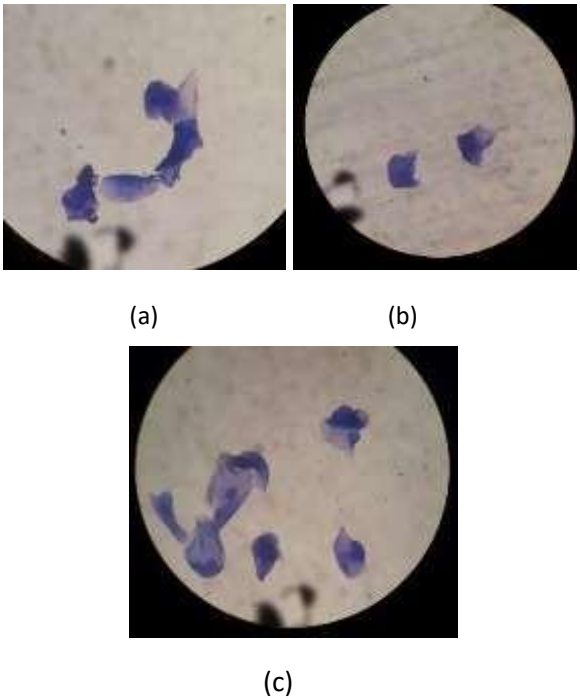

Figure 2. Documentation of first vaginal smears. Slides were dominated by cornified/superficial (a,b) and intermediate cells (c) at 40× magnification (personal documentation by *handphone* Samsung®A5 camera).

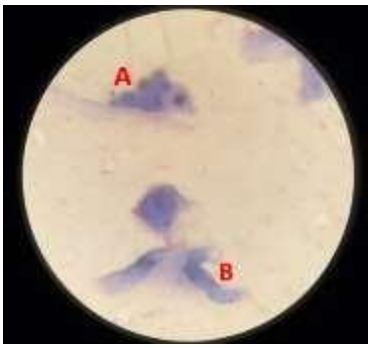

Figure 3. Documentation of second vaginal smears. Slides were dominated by basal cells and parabasal cells (A) and intermediate cells (B) at 400× magnification (personal documentation by *handphone* Samsung®A5 camera).

5% to 40% of total cell population and intermediate cells decrease progressively from 45% to 6%. At the 4th – 7th day of follicular phase while superficial cells

stay at range from 40% - 50%. Parabasal cells are not found in the smears during follicular phase. Parabasal cells generally low through the cycle, 1% - 6% according to [42] or <10% according to [45] from total population of the cells on the smears. The explanation from previous research [42] are the follicular phase is defined as the period of time when the estradiol-17β concentrations in plasma exceeded 20 pg/ml from the base levels (11.7 pg/ml). The mean length of the follicular phase is around 7.4 days and ranged from 3 – 16 days.

This present study showed there was no neutrophil (-) infiltration on vaginal smears. In this case, it could be happened due to minimum time during sample collection. All sample collection had to be done under <3 minutes to minimize the stress of the tiger. Neutrophils are observed on vaginal smears in other literatures (13,14,39,40). According to previous studies, neutrophil densities increase from little (+) to medium (++) at the onset of proestrus (13). Neutrophils may be seen in the first few days of proestrous but cannot be found in the middle of proestrous (41). Few neutrophils (+) presence at the late proestrous. At estrous period, neutrophils begin to reappear. Neutrophils are dense (+++) at metestrus of the estrous cycle. Neutrophils begin to present in low densities during anoestrous [13,39,43]. Neutrophils are observed in vaginal epithelial tissue at every period confirmed by histological evidence [44]. Neutrophils are relatively delicate cells and can easily rupture during sample collection or processing vaginal smears [14]. Neutrophils may lost of sight during vaginal smears observation under the microscope.

Luteal phase and follicular phase of the female tiger could be determined on this present study. However, contact or interaction between human and tiger should be minimized on this research to sustain the tiger's wild behaviour. Sample collection was performed twice in this research. Sample collections on daily basis for future researches were recommended to detect estrous cycle (proestrous, estrous, metestrous, and anestrous).

Variety length of estrous cycle has been reported in other literature by [46,47,48,49]. The usual oestrous cycle length of Bengal tiger is 25 days based upon behaviour observations and hormonal data [47]. The longest estrous cycle length of Sumatran tiger is 58.3 ± 2.7 days based on fecal progesterone (P4) and sexual behaviour observations in Japan has been reported by [48].

The female Sumatran tiger at Tambling Rescue Centre showed different phase within 6 months of interval. Contrary to [47] findings, Siberian tiger has anestrous period (which is luteal phase) up to 8 months period of time. Based on the usual estrous cycle length (25 days), this research predicted the tiger had passed 7 estrous cycles.

This female tiger habitat was conditioned in tropical season. Both cages (solitary cage and habitual cage) were designed partly covered so the tiger could access freely to direct sunlight or sheltered from the sunlight. The individual cage was covered by the roof and the habitual cage was covered with natural trees. From the earlier researches, tiger's estrous cycle and breeding season are synchronized by photoperiod [47,48,49,50]. Tiger's reproduction is seasonal as well as clouded leopard, Palla's cat and snow leopard species. Other felids species such as fishing cats, pumas, ocelots, margays, leopards and captive lions are not influenced by season [48]. Estrous cycles of the female Felidae are highly variable. The estrous cycle of female felids may be influenced by seasonality factor, dietary factor, housing, and captivity-related stress [49].

P_4 and estrogen hormones have been recorded for reproductive analysis on animals [47,49]. Hormonal assays have its own challenges. Results from hormonal assays may be influenced by degradative activity of the bacteria on the feces that affect to the P_4 metabolites, transportation condition, sample storage, extraction method, sensitivity and specificity of the assays [50]. Vaginal smears are fine aid in the diagnosis of the period of estrous cycle in many species and reflects the effect of estrogen-progesterone hormones interaction on the female reproductive tracts [12]. Vaginal smear method remains as a proper method or the gold standard method in life animals than others verified method to detect estrous cycle [41].

3.2. Combining neutrophil blood counts with vaginal smears examination

The first neutrophil blood count (Figure 4) resulted 78 cells on first collection and 55 cells on the second collection. Elaborated with medical check- up result on the female tiger by the veterinarian team, the tiger was on a healthy condition with no clinical symptoms. There is a minor difference compared to other literatures. Other literatures show neutrophil values ranged from 57 – 75 cells on wild ranging Bengal tiger with anaesthesia [51]. Female captive tiger has neutrophil values ranged 63.58 ± 0.48 cells without any anaesthesia [52]. Anaesthesia not only has anaesthetic and analgesia effects but also

immunomodulatory effects. Neutrophils as innate and adaptive immunity agents [53,54] are affected by ketamine [55]. Ketamine supresses neutrophil chemotaxis (neutrophil migrations) leads to immunosuppression effect [55,56].

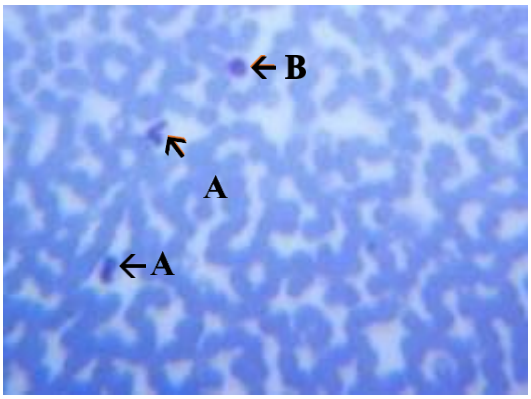

Figure 4. Neutrophil blood count. (A) Neutrophils with segmented nuclei and (B) lymphocyte on blood smears at 400× magnification (personal documentation by *handphone* Samsung®A5 camera).

Both [51] and [52] researches, the tiger bloods are added to anticoagulant agent on sample collection. A possible explanation is both neutrophil values from those literature were using EDTA. Blood smears were fixed using methanol directly after the blood collection in this research. Time storage of anticoagulant EDTA on blood sampling effects the blood count. Prolonged storage of peripheral blood cell in EDTA produces various artefacts. Neutrophil percentage decreases between 1 hour, 4, 8, &12 hours storage at the room temperature (57,58). Low leucocyte counts due to leucocyte aggregation induced by EDTA has been reported by (59,60). Neutrophil count with citrate coagulation shows 6,26 × 10^9/L, while EDTA coagulant shows 4,8 × 10^9/L (60).

Neutrophil blood count was higher on the 1st sample than the 2nd sample. Other researcher [17] provides evidence by immunohistochemical data of neutrophil counts in the blood were significantly high at day one (or 1st day) compared to 2nd day of the estrous cycle. Their findings suggest there is possibility the neutrophils from the blood translocate to the female reproductive tract (FRT) such as endometrium on 2nd day of the estrous cycle. Another research [18] supports those statements by reviewing (1) the contribution of sex hormones in regulating the functionality of neutrophils, (2) the contribution of neutrophils during fertilization process, pregnancy process and, controlling infectious diseases (caused by viral/fungal/bacterial). Lower numbers of neutrophils on FRT are observed at ovulatory/follicular phase. Increased numbers of neutrophils on FRT are observed at post-ovulatory phase. Cyclic steroid sex hormones regulate the permeability of the FRT and then allowing neutrophil to access into FRT.

Neutrophils number decline in the vaginal lumen during the follicular phase of estrous cycle. Neutrophils in the vaginal lumen are characterized by the elevated estradiol (E_2) levels, and then disappear at the onset maximum peak of E_2 level of the ovulatory phase, and finally neutrophils elevate again during the luteal phase. P_4 levels are at peak during the luteal phase of estrous cycle. High level of E_2 level throughout the ovulation period may inhibit neutrophil-mediated immunity. E_2 and P_4 affect epithelial cells [61,62]. The cyclical wave (physiological regulation) of neutrophil's number in the vaginal lumen migration is initiated by chemokines [61,62] and correlated with circulating E_2 hormone levels [44].

Brown (2006) [48] summarized endocrinology of nondomestic felids. Female cycles in general are within 2 – 4 weeks interval with estrous period lasting from 3 – 10 days. Estrogen's surges from fecal sample collection can distinguish estrous period from intrerestrous period in female reproductive cycle. In the other hand, pregnancy diagnosis based on P_4 hormone level analysis during the 1st half of gestation period is not possible due to indistinguishable hormone level to the luteal phase profiles. Technically it possible to diagnose gestation or pregnancy if the P_4 is still elevated after 40 days post-copulation [47,63]. P4, testosterone, and, androstenedione hormone metabolites are significantly increased on the gestation period compared to the lactation period on the female tiger [64].

In this present research, neutrophil blood counts were integrated with vaginal smears examination to complement the detection of estrous cycle. Neutrophil blood count was higher (77 cells) when the vaginal smears were dominated by intermediate and superficial cells. Contrast to the 1st sampling, neutrophil blood count was lower (55 cells) when the vaginal smears were dominated by basal and intermediate cells on the 2nd sampling. It indicated that neutrophil on the blood was higher during follicular phase and lower during luteal phase. This research aligned with previous research [17].

Neutrophils play important role in both innate and adaptive immunity. Neutrophils are the 1st type of

leucocytes to arrive at affected tissues and as a microbicidal agent [53,54]. The presence of neutrophils at FRT are to aid the breakdown of endometrial tissue of FRT with elastase and to elevate the innate immunity defense mechanism as the epithelial barrier is disrupted. Commensal organisms, growth factors and sex hormones contribute to regulate cells of the innate and adaptive immune systems against potential pathogens. Regulation of immune systems (innate and adaptive immunity) protects the FRT [65].

During at the end of ovulatory phase, estrogen reduces the membrane permeability of the FRT. Then the migration of neutrophil to the FRT decreases to permit sperm survival and mobility in the FRT. It leads in increased risk of infection [18]. Medication from E_2-based or deregulation of P_4 expression may compromise the vaginal immunity and making FRT more vulnerable to pathogens [66].

3.3. Detecting Breeding Time to Maintain Tiger Population

This research showed vaginal smears using cotton swab can be applicated on big cats such as tiger. Consistent to (11) and (67) studies, this method was effective under field conditions. Neutrophil blood count could be a complementary method on the vaginal examination to identify estrous phase. Behavioural observation may be collaborated with vaginal smears and neutrophil blood count method in the future researches.

Vaginal smears collection and evaluation/assessment techniques required practice and proper training. Previous research [14] mentioned those training is easily mastered. This research also suggests to practice vaginal smears collection, blood sampling, and evaluation at the laboratory before performing those techniques in the remote setting/area. Proper training and practice lead to consistent and reliable data.

Information of optimum time for breeding might be useful for maintaining tiger population. The female tiger on this research had history of mating with free- ranging male tiger on the Tambling Rescue Centre and successfully delivered tiger cubs in 2012. Both natural mating and ARTs required estrous detection for breeding programs. Estrous detection for breeding is already popular on pet animals [5,43] and farm animals [6,7]. Maintaining tiger's population through breeding programs is a part of conservation efforts.

Reproductive knowledge of basic biology to advance biotechnologies in Felidae have been continually researched and studied for conservation. The zoos have an important contribution in the felid conservation by assisted reproductive biotechnology (ARTs) protocols to create ex-situ breeding and management programs. In the long run, ex-situ conservation also provides knowledge to sustain genetic diversity and then finally sustain the population of endangered felid species [68]. Reproductive aspects on wild female tiger have been reported by [69] and gene flow by [70]. Both of ex-situ and in-situ conservation must collaborate to maintain biodiversity conservation [71,72].

4. CONCLUSION

Vaginal smears and neutrophil blood count can be an alternative method to determine estrous phase beside hormonal assays. Neutrophil blood count can act as a complement to vaginal smear method in detecting estrous phase. Neutrophil blood count is higher during follicular phase. In the contrary, neutrophil blood count is lower during luteal phase. This research shows that it is possible to perform those methods on a tiger, especially in the remote area.

ACKNOWLEDGMENT

We would like to express our appreciation to Tambling Wildlife Nature Conservation, Balai Konservasi Sumber Daya Alam & Lembaga Ilmu Pengetahuan Indonesia for supporting this research.

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