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Fecal Estradiol and Progesterone Measurement for Determining Ovarian Function of Indonesian Spotted Buffaloes

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Abstract: Spotted buffalo is an exotic animal in Tana Toraja, South Sulawesi, Indonesia. Population of this animal is predicted to decline due to high rate of male to be slaughtered at traditional ceremonies as well as female reproductive characteristic. The aim of the study was to observe ovarian function of this animal through fecal estradiol and progesterone measurements. Fecal samples from four adult females were collected every two days for 60 days, then were analyzed using ELISA for estradiol and progesterone. Vulval swelling and sexual behavior were also observed. The results showed fluctuations in both estradiol and progesterone profiles in all females and represented ovarian cyclicity particularly in two females with the length of follicular phase 9-11 days and luteal phase 11-13 days. Estradiol concentrations were ranging from 51.94 ng/g DW to 224.03 ng/g DW during follicular phase and the minimal concentration of progesterone was 0.6 ng/g DW and reach the peak at 11.7 ng/g DW during luteal phase. In other two females, in the contrary, fluctuation of the profile did not seem to be clear and the concentration of both estradiol and progesterone were too low compared to the others. No genital swelling and sexual behavior could be observed.

Key words: Spotted buffalo · Ovarian function · Fecal estradiol · Fecal progesterone

INTRODUCTION

Spotted buffalo (*Bubalus bubalis*) is the most important animal in the life and culture of the Torajans [1]. The relationship between spotted buffalo and the Torajans is very close. The existence of this animal becomes very important in every traditional ceremony, either for burial or for celebration. This animal is thought to bring fortune and is usually used for veneration in those ceremonies. At the funeral, the type and the amount of male spotted buffalo sacrificed can represent the level of the host social life [2]. The different shapes of the horns, the different colors and various marks each animal are all beings used for judging the value and price of the animal. It is not surprising then that this buffalo became the most highly valued animal in this region and since this animal is always included in important rituals, the demand for it exceeded the ability of the people of Tana Toraja to raise and to supply that demand. According to the local people believe, spotted buffalo can only be survive in its natural habitat, which is in Tana Toraja. The farmer put a special treatment for this animal. The male is maintained separated from the female to prevent it from reproduction activities as the Torajans believe that if the male does sexual activities, this male will get wild and uncontrollable [3].

The population of the spotted buffalo is thus predicted to decline in the future because of the high rate of the male to be slaughtered at the tradition ceremonies (~ 300 animal/ year) as well as male to be isolated and castrated. Moreover, to fulfill the high demand of the Torajan for meat consumption (~ 120 animal/year), the population also need to be increased. As an exotic species in Toraja, the population of spotted buffalo,

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therefore has to be maintained or even to be increased through natural breeding and/or artificial breeding program.

Unlike cattle, to breed the buffalo is relatively difficult as female buffalo has concealed estrous signal. Reproductive efficiency is the primary factor affecting productivity and is hampered, in female buffalo, by the late attainment of puberty, long postpartum anoestrus and subsequent calving interval [4]. Thus, to achieve a success breeding program for this species there is an urgent need to acquire basic information on the reproductive biology both on the male and female sites which are no doubt very pivotal point for assessing the function of reproductive system of both sexes which reflect their fertility.

The methodology used for evaluating the ovarian function of this animal was non-invasive methodology through fecal steroid measurement and this is the first in spotted buffalo. Non-invasive approach was used in this research because the farmers are very protective to their animals and the chance to get permission for collecting blood sample as well as getting touch with the animal was too little. All together with the estrous signal that is probably exhibited by the female will be important information for further application of reproduction technology.

The aim of this research was to describe the reproductive status of the female spotted buffalo through the analysis of fecal estradiol (E2) and progesterone (P4), including determination the length of the phases comprises the estrous cycle and the prediction of optimal mating time. Proper estrous detection is actually not easy but important point to be assessed because proper estrous management facilitates the use of artificial insemination. Essentially similar basic approaches are being used in buffaloes as there are in cattle. Apart from the scientific information, it is hoped that the findings will has positive impact for farmers in Toraja in order to support the existence of this animal.

MATERIALS AND METHODS

Animal Housing: Spotted buffaloes used in this study were four adult females with different spot patterns and prices. Those females were maintained at four different areas in their natural habitat, which is at Sad'dan Village, Sad'dan Malimbong Distric, North Toraja Regency, South Sulawesi, Indonesia. Those females were maintained at four different family farms and the type of cages were individual cage and big cage for 2-3 individuals. Feedstuff given was grass, that was supplied twice a day and access to water was *ad libitum*. Sometime the animals were left out to the landgrass during the day for grassing.

Fecal Samples Collection: 5-10 g fecal sample was collected in a plastic tube every two days at 7.00 h -8.00 h in 45 days period and following collection, samples were immediately stored at -20°C without preservative until assayed. Visual inspections of the genital swelling and other estrous sign exhibited by the animals were also carried out at the same time as sample collection.

Sample Preparation: Prior to analyze, the samples were extracted as described by [5] for estradiol (E2) and progesterone (P4) measurements. A total amount of fecal samples collected were first lyophilized and the resulting dried pellets were pulverized and extracted with 3 ml of 80% methanol in water by vortex for 10 minutes followed by centrifugation at 2200 x g for 10 minutes. Supernatant was decanted into a clean glass tube and after appropriate dilution in assay buffer (5.96 g Na_2HPO_4, 8.50 g NaCl and 1 g bovine serum albumin (BSA) Fr. V in 1 L H_2O, pH 7.2) was taken directly to assay.

Estradiol and Progesterone Analysis: Microtiter plate EIAs for assay was supplied by Diagnostic-Related Group (DRG) Instruments GmbH, Germany. E2 and P4 were analyzed using E2 and P4 EIAs Kit and procedures of assay were performed as instructed by the DRG's Kit manual. Parallelism test was also performed in replicate dilutions using the same Kits to validate the assays used. The samples were diluted in assay buffer with a certain dilution depending on the reproductive status. Cross reactivity of the E2 assay for estradiol-17β, estriol and estrone were 100%, 0.05% and 0.2%, respectively. Specificity of the P4 assay tested for progesterone and 17αOH progesterone gave cross reactivity of 100% and 0.30%, respectively. Sensitivity of E2 assay was found to be 9.714 pg/mL and 0.045 ng/mL for P4 asaay. Intra and inter-assay coefficient of variations were 4.66% and 7.79% for E2 assay, whereas for P4 assays were 6.42% and 6.63% All the validation data rather than parallelism data were supplied by the KIT manufacture.

Data Analysis: Profiles of E2 and P4 obtained were used to particularly evaluate ovarian function and also reproductive pattern, length of follicular and luteal phases and the correlation with genital swelling and other estrous sign observed. Luteal phase was defined as the threshold

value of three standard deviations above the mean of the preceding follicular phase values to assess timing of the first postovulatory increase in fecal progestin concentration. An increase in concentrations above this threshold value was taken to indicate a statistically significant rise (P<0.01) [6]. The same evaluation was applied for determining follicular phase through E2 profile obtained.

RESULTS

The four females of spotted buffalo used in this study represented three types of speck patterns: 1) *Saleko* type, buffalo which has black and white coloration almost the same portion in the skin, with specific pattern of specks throughout their body. The pictures of male and female *Saleko* can be seen in Figure 1. 2) *Todik* type (Figure 2), which is a black buffalo with star-looked white spot on the forehead and 3) *Bonga Ulu* type (Figure 3), buffalo which has white color only on the head whereas the neck and the whole body is black.

Prior to hormone measurement, parallelism test was carried to validate the assay and to find the appropriate dilution of the samples. Serial dilutions of fecal extracts were analyzed using estradiol and progesterone EIAs KIT produced by DRG GmbH, separately and the results gave displacement curves parallel to those of both estradiol and progesterone standards.

All figures presenting the profile of immunoreactive estradiol and progesterone in individual female showed fluctuation in both estradiol and progesterone profiles, however only the profile of two females (Figure 4 and 5) seems to be reflecting normal ovarian cycle with clear follicular and luteal phase. Female #1 (*Saleko* type) in particular showed discrete pattern of luteal phase in its P4 profile and basal progesterone level that was presumably the follicular phase. Although there was an increase in the hormone level for other two female (Figure 6 and 7), but it seems to be too low to reflect the pattern of follicular as well as luteal phase.

The lowest concentration of fecal estradiol of the cyclic female was 51.94 ng/g of dry weight of feces (DW) and reach the peak at 224.03 ng/g DW, whereas the lowest concentration of fecal progesterone was 0.6 ug/g DW and reached the peak at 11.7 ug/g DW. The length of luteal phase resulted from both estradiol and progesterone patterns was ranging from 11-13 days. As also seen in both hormone patterns, the length of estrous cycle could be predicted for 20-24 days with 9-11 days of follicular phase. Basal progesterone level of *Saleko* type (Figure 4)

A

B

Fig. 1: Spotted buffalo (*Saleko* type): a. Male, b. Female

Fig. 2: Spotted buffalo (*Todik* type)

Fig. 3: Spotted buffalo (*Bonga Ulu* type)

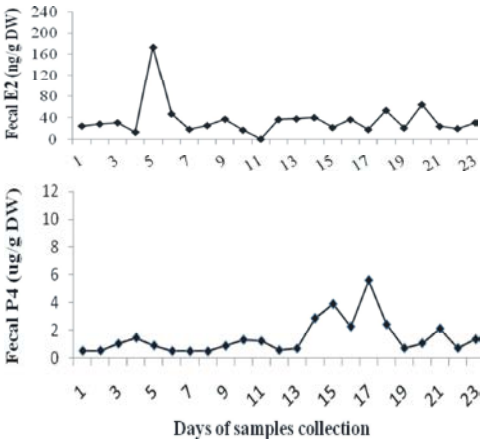

Fig. 4: Fecal E2 and P4 profiles of female spotted buffalo # 1 (*Saleko* type)

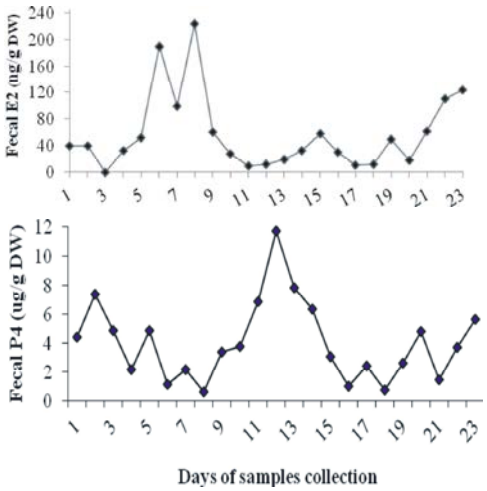

Fig. 5: Fecal E2 and P4 profiles of female spotted buffalo # 2 (*Todik* type)

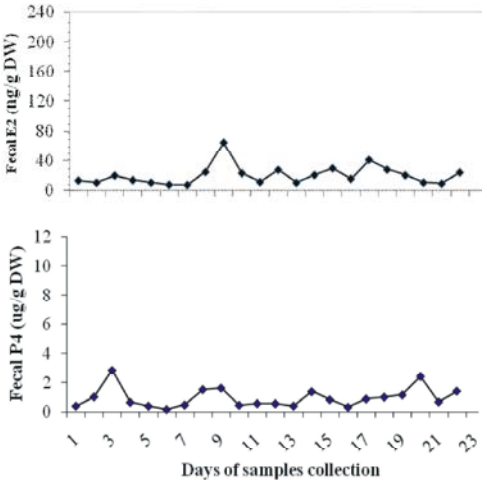

Fig. 6: Fecal E2 and P4 profiles of female spotted buffalo # 3 (*Todik* type)

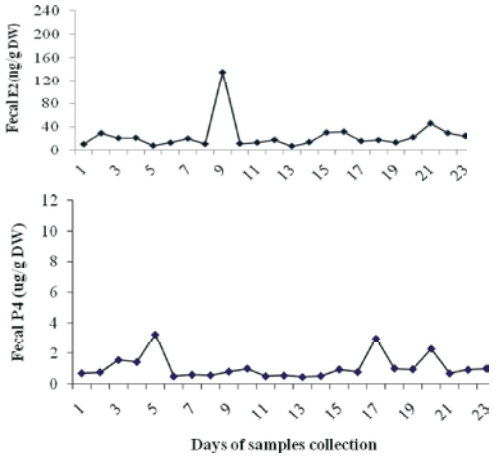

Fig. 7: Fecal E2 and P4 profiles of female spotted buffalo # 4 (*Bonga Ulu* type)

seems to be longer that presumably animal was in extended follicular phase at the time fecal samples collection. In contrast, the progesterone profile of the *Bonga Ulu* and *Todik* type females (Figure 6 and 7) remained low throughout the study and it indicate inactive ovaries.

No estrous sign could be observed through visual inspection of vulvar swelling nor sexual behavior because there was no chance to put male and female together. In two females (#1 and #2 in Figure 4 and 5), however, a slight swelling of the external genitalia was observed for 1-2 days.

DISCUSSION

Spotted buffalo is a member of swamp buffalo which is almost all of these species are raised in small farms for working and meat. Traditional reproduction management dominates the buffalo production system [7]. Similar situation was also found in Tana Toraja, South Sulawesi, Indonesia. In contrast with the male which is treated with a special care, female spotted buffaloes in Tana Toraja are freely grazed on natural grassland and not in a proper care. Observation and recordings on their reproductive performance are very limited. Breeding selection is almost negligible, except that farmers allow the mating of the female to excellent ordinary bull rather than to the spotted male. Based on empirical observation, it was found that the spotted buffalo was not always produced from the spotted buffalo parents. Sometimes the mating process between ordinary and spotted buffaloes or even between the same ordinary buffaloes could yield progeny which had spot pattern (personal communication).

This phenomenon will be very challenging to be explored and study through the application of assisted reproductive technologies such as artificial insemination in order to obtain offspring which has preferred quality and specks as the parent bull. To prepare animals for that purpose, basic reproductive endocrinology of the female need to be assessed for describing the condition of its reproductive system.

Fecal progesterone metabolite monitoring techniques were validated for female spotted buffalo as difficult to have access for blood collection. Longitudinal fluctuation profiles obtained were consistent with typical luteal phase patterns in cyclic female and corresponded closely with changes in serum progesterone of cyclic buffalo [8, 9]. Moreover, it is also reported that fecal progesterone concentration was clearly correlated with that of in blood, therefore this non-invasive method is a valuable tool for determining the luteal status and such information may be useful for developing estrus synchronization in buffalo [10].

According to the result obtained from this research, estrous sign as well as estrous behavior was difficult to be observed and this consistent with the statement that buffalo estrus behavior is less intense than that of cows and is consequently much more difficult to detect [11]. Mucosal vaginal discharge, swollen vulva, mounting behavior (far less frequent than in cattle) and the standing reflex are the main signs of estrus. Unfortunately, the chance to observe sexual behavior between two sexes could not be performed in females observed.

Compare to plasma progesterone level in cattle, the concentration of this hormone was less than 1.0 ng/ml from 2 days before estrus to 3 days after estrus and gradually increased from 4 days after estrus as the corpus luteum became functional [12]. It reached a maximum of 8.0 to 10.0 ng/ml at 11 to 15 days after estrous (the "plateau progesterone" period) and then declined (the "falling progesterone" period) to basal levels before the next estrus and ovulation. During the same period, plasma progesterone levels increased from 0.21±0.01 ng/ml at day 0 to a peak of 3.30±0.72 ng/ml on day 13, declining sharply by day 5 [13]. The pattern is similar with that of found in cyclic female observed. Instead of progesterone, other reproductive hormone play in pivotal role in estrous cycle is estradiol, so that it would be worth if estradiol measurement was also included in the research for assessing various events occurring throughout the estrous cycle [14].

Other researcher noted that differences in progesterone concentration have sometimes been suspected to arise from differences in ovarian size. The lesser responsiveness of the ovaries to gonadotrophic hormone during formation of a corpus luteum might also result in a smaller corpus luteum. Variation of individual in ovarian dynamics could be revealed from this data and further observation is actually needed to be carried out such as implementation of rectal examination or ultrasound application for further treatment if any problem encountered the reproductive system [15].

In general, reproductive performance of animals is affected by many factors such as nutrition, management and environment [16]. Fertility in water buffalo (*Bubalus bubalis*) is considerably lower than that in cattle (*Bos taurus* and *Bos indicus*). Poor breeding efficiency is attributed to late onset of puberty, seasonality, poor estrus expression and long calving intervals. Accurate estrus detection is a prerequisite for efficient reproductive management particularly in determining optimal mating time. The steroid metabolite profile is therefore can serve as a good tool in supporting natural or artificial breeding program of spotted buffalo.

From the results obtained, it can be concluded that in general, fecal progesterone measurement could be used as a reliable tool for assessing female reproductive status in spotted buffalo but thorough observation need to be applied such as ultrasound examination and sexual behaviour between male and female rather than estrous sign observation to confrim individual ovarian dynamics in the future.

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