

## Using Milk Progesterone Assay at the time of Oestrus and Post-mating for Diagnosing Early Pregnancy in Anatolian Water Buffaloes

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**Abstract:** Milk progesterone level is a good marker to evaluate the functional status of the corpus luteum in all farm animals. The present study was conducted to monitor changes in milk progesterone concentrations at the time of oestrus and post-mating for diagnosing early pregnancy in Anatolian water buffaloes.

Twenty-one buffaloes reared at the Kocatepe Agricultural Research Institute, Afyon, Turkey, were used. Milk samples were collected on Days 0 (DO: oestrus-mating), 11, 19, 21 and 24, and then every 3-4 days until Day 45. They were stored at 4 °C and analysed by the enzyme immunoassay (EIA) method. Rectal palpation was performed on Day 60 to confirm pregnancy.

Although 16 animals had elevated levels of progesterone on Day 19 and Day 24, pregnancy was only confirmed in 12 of them on Day 60. Although the other 4 buffaloes had elevated levels of progesterone and did not return to oestrus during Day 19 and Day 24, they were confirmed non-pregnant on Day 60. Two of these non-pregnant buffaloes with elevated progesterone levels had increased progesterone levels even on Day 45 and did not return to oestrus. In the remaining 2 non-pregnant buffaloes with elevated progesterone levels, progesterone concentrations declined gradually (1.2 ng/ml on Day 31 and 0.88 ng/ml on Day 45).

The other 5 buffaloes were diagnosed non-pregnant based upon the milk progesterone assay. Mean progesterone concentration in these non-pregnant buffaloes was  $0.88 \pm 0.37$  ng/ml between Days 19 and 24. The accuracy rate, sensitivity, specificity and the detection rate of pregnancy and non-pregnancy of progesterone assay were 81.0, 100.0, 56.0, 75.0 and 100.0%, respectively.

In conclusion, milk progesterone assay is a fast and reliable tool to detect oestrus time and non-pregnant buffaloes with a 100.0% accuracy rate. In addition, this assay may also give an idea about early pregnancy during Days 19-24 of gestation and may be used to predict some reproductive disorders which cause infertility and economic losses in Anatolian buffaloes.

**Key Words:** Anatolian buffalo, milk progesterone, early pregnancy

### Anadolu Mandalarında Süt Progesteron Analizlerinin Östrus Anında ve Aşım Sonrası Erken Gebelik Teşhisinde Kullanılması

**Özet:** Süt progesteron seviyesi tüm hayvanlarda corpus luteumun fonksiyonel durumunu gösteren bir araçtır. Bu nedenle sunulan çalışma, Anadolu Mandalarında östrus anında ve çiftleşme sonrasında süt progesteron konsantrasyonlarının erken gebelik teşhisinde kullanılabilirliğini belirlemek için düzenlendi.

Araştırmada Afyon Kocatepe Tarımsal Araştırma Enstitüsünde bulunan 21 manda materyal olarak kullanıldı. Hayvanlardan süt örnekleri 0. (östrus-çiftleşme), 11., 19., 21., 24. günlerde ve ayrıca 45. güne kadar her 3-4 günde bir toplandı. Örnekler 4 °C'de saklandı ve daha sonra enzim immunoassay (EIA) metodu ile analiz edildi. Hayvanların gebelikleri 60. günde rektal palpasyon ile kontrol edildi.

Analizler sonrasında 16 mandanın süt progesteron seviyeleri 19-24. günler arasında yüksek bulunmasına rağmen, sadece 12'sinin rektal muayenede gebe olduğu belirlendi. Geriye kalan 4 mandanın ise östrusa gelmedikleri ve rektal muayene ile gebe olmadıkları tespit edildi. Bunların 2'sinde progesteron seviyesinin 45 gün süresince düşmediği gözlenirken, diğer 2'sinde ise süt progesteron konsantrasyonlarının sırasıyla 31. (1,2 ng/ml) ve 45. (0,88 ng/ml) günlerde düştüğü belirlendi.

Çalışmada değerlendirilen diğer 5 mandanın 19. ve 24. günler arasında süt progesteron konsantrasyonları  $0,88 \pm 0,37$  ng/ml olarak tespit edilirken hayvanların gebe olmadığı belirlendi. Bunun yanı sıra progesteron testinin doğruluk, duyarlılık, özgüllük, gebe olanları ve olmayanları belirleme değerleri ise sırasıyla % 81,0, 100,0, 56,0, 75,0 ve 100,0 olarak elde edildi.

Sonuç olarak, süt progesteron analizlerinin Anadolu Mandalarında östrus zamanını ve gebe olmayanları % 100,0 doğruluk oranıyla belirleyebilen hızlı ve güvenilir bir araç olduğu düşünülmektedir. Ayrıca bu testin erken gebelik ve mandalarda infertiliteyle birlikte ekonomik kayıplara yol açan bazı reproduktif sorunlar hakkında ön fikir verebileceği kanaatine varıldı.

**Anahtar Sözcükler:** Anadolu mandası, süt progesteron, erken gebelik

## Introduction

Anatolian water buffalo is a native breed and is commercially very important for the local milk-cream industry in Afyon, Turkey. However, poor buffalo management has resulted in various reproductive problems including unknown reproductive disorders causing infertility and thus economic losses in the milk industry.

The milk progesterone level is a good marker to determine the functional status of the corpus luteum and ovarian activity in all farm animals. Monitoring milk progesterone concentrations gives practical results to improve reproductive functions and may also detect reproductive disorders such as lack of cyclicity (anoestrus), silent heat, cystic ovarian disease, persistent corpora lutea and irregular cycles (1-4). A number of studies have shown that the progesterone level could give reliable results for detecting oestrus time (5-7) and for the early estimation of pregnancy in dairy cows and buffaloes (3,8). The accuracy of the milk progesterone test was reported to be 75.0% for early pregnancy diagnoses and 100.0% for non-pregnancy estimation on Day 22 of gestation (3,5).

The present study was conducted to detect the changes in progesterone concentration by using milk progesterone assay in Anatolian buffaloes at the time of oestrus and post-mating. In addition, it is planned to assess the prospects of using milk progesterone assay for the detection of pregnancy.

## Materials and Methods

### Animals and management

Twenty-one lactating and cycling Anatolian buffaloes reared by the Kocatepe Agricultural Research Institute, Afyon, Turkey, were used during the late spring and early

summer. The buffaloes were housed in a semi-open yard and fed with concentrate mixture and dry alfalfa according to their body weight and milk production. All animals were checked for oestrus 3 times a day for 30 min with a fertile Anatolian buffalo bull and mated if they were on oestrus.

### Milk sampling and storage

Milk samples (5 ml/animal) were collected at Day 0 (DO: oestrus-mating), Day 11, in the middle of the oestrus cycle, and on Days 19, 21 and 24. Collection of milk samples continued from Day 28 to Day 45 at 3-4 day intervals to estimate reproductive failures by using milk progesterone levels. All milk samples were preserved with potassium dichromate tablets (Merck) and were stored at 4 °C until assayed.

### Milk progesterone assay

Milk progesterone concentrations were measured by enzyme immunoassay (EIA) as previously described by Van de Wiel and Koops (9). Briefly, fat was separated from the milk samples by centrifuging at 3000 rpm for 30 min at 5 °C. Then wells were coated with anti-rabbit/goat IgG (1 µg/well). Progesterone standards were prepared in defatted milk as 0 ng/ml, 0.3 ng/ml, 0.6 ng/ml, 1.25 ng/ml, 2.5 ng/ml, 5 ng/ml, 10 ng/ml and 20 ng/ml. Aliquots of 20 µl from both standards and samples were placed in each well. Primary antibody (100 µl) was added to each sample. Then 50 µl of enzyme (Horseradish Peroxidase, RZ 3, Boehringer) was added. Plaques were incubated at 37 °C for 2 h and at 4 °C overnight. After incubation, the wells were rinsed with Tween 80 and air-dried. Aliquots of 150 µl of substrate (TMB: tetra methyl benzidine, 3,3',5,5'-tetramethylbenzidine, Sigma) were added and incubated in the dark for 40 min. The reaction was finally stopped by adding 50 µl of H<sub>2</sub>SO<sub>4</sub>. All samples were read at 450 nm and data were analysed using a standard curve.

### Rectal palpation and checking the accuracy rate of milk progesterone analysis

Rectal examination was performed on Day 60 to confirm pregnancy. The accuracy rate, sensitivity, specificity and the detection rate of pregnancy and non-pregnancy for the milk progesterone assay were determined as reported by Taverne et al. (10) as follows:

Correct-positive diagnosis:	a
False-positive diagnosis:	b
Correct-negative diagnosis:	c
False-negative diagnosis:	d
The total number of animals:	e
Accuracy rate:	$a + c/ex100$
Sensitivity:	$a/a + dx100$
Specificity:	$c/c + bx100$
Detection rate of pregnancy:	$a/a + bx100$
Detection rate of non-pregnancy:	$c/c + dx100$

### Results

In 16 buffaloes, the progesterone level was elevated between Days 19 and 24. However, only 12 of these 16 buffaloes were determined to be pregnant on Day 60. The progesterone concentration in pregnant Anatolian buffaloes (n: 12) was  $0.97 \pm 0.42$  ng/ml at the time of oestrus,  $7.99 \pm 2.95$  ng/ml on Day 11, and  $8.04 \pm 2.94$  ng/ml on Day 21 post-mating (Figure 1 and Table). Four buffaloes with elevated levels of progesterone were confirmed non-pregnant on Day 60. Two of these non-pregnant buffaloes with elevated progesterone levels had maintained the increased progesterone concentration over 45 days (Figure 2). On Day 60, however, luteal

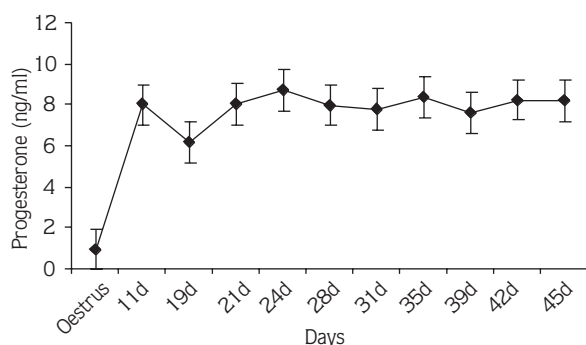


Figure 1. Mean progesterone concentration in early pregnant buffaloes (n: 12).

Table. Mean progesterone levels of pregnant animals (n: 12) palpated by rectum on Day 60 of gestation.

Days	Progesterone (ng/ml)
0 (Mating day)	$0.97 \pm 0.42$
11	$7.99 \pm 2.95$
19	$6.15 \pm 1.87$
21	$8.04 \pm 2.94$
24	$8.68 \pm 3.57$
28	$7.98 \pm 4.02$
31	$7.80 \pm 2.12$
35	$8.34 \pm 2.56$
39	$7.62 \pm 3.34$
42	$8.23 \pm 2.63$
45	$8.18 \pm 2.02$

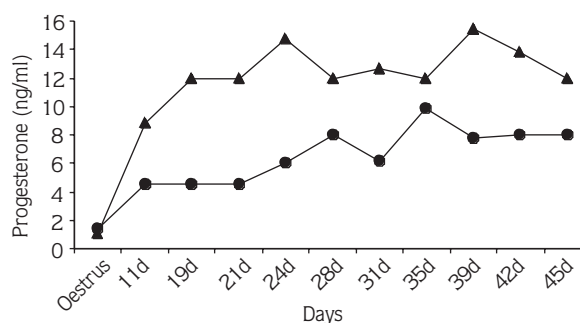


Figure 2. Elevated levels of progesterone concentration over 45 days in 2 non-pregnant buffaloes.

structures were palpated in these 2 animals, and no differential diagnosis could be performed between persistent corpora lutea and a luteinised cyst. In the 2 other non-pregnant buffaloes with elevated progesterone levels, progesterone concentrations decreased gradually, from 1.2 ng/ml on Day 31 to 0.88 ng/ml on Day 45 (Figure 3), and then oestrus symptoms were observed.

In 5 buffaloes the mean progesterone concentration was  $0.88 \pm 0.37$  ng/ml on Days 19 and 24 (Figure 4) and pregnancy did not occur, which was confirmed on Day 60 by rectal palpation. At the end of their cycles, they returned to oestrus within the normal range of 19-24 days.

The accuracy rate, sensitivity, specificity and the detection rate of pregnancy and non-pregnancy for milk progesterone test were calculated as 81.0, 100.0, 56.0, 75.0 and 100.0%, respectively.

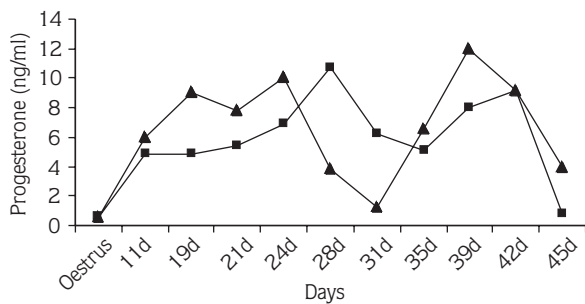


Figure 3. Progesterone concentration decreased on Day 31 and 45 for two buffaloes

## Discussion

The water buffalo has a poor reproductive performance, which is mostly attributed to delayed maturity, poor clinical signs of oestrus and long calving intervals. Seasonality of buffalo breeding (11) results in a milk supply that exceeds demand in summer and autumn and diminishes significantly in winter and spring. The reproductive efficiency of water buffaloes must be improved to prevent economic losses. One practical approach is to monitor the reproductive status of the buffaloes. We found that milk progesterone assay may help to estimate early pregnancy. In addition, milk progesterone assay is a fast and reliable tool with which to detect non-pregnant buffaloes; thus, it helps with the measures necessary to improve reproductive efficiency.

The mean progesterone concentration of pregnant Anatolian buffaloes ( $n: 12$ ) was  $0.97 \pm 0.42$  ng/ml on oestrus (Figure 1 and Table). As previously reported by Aksoy and Tekin (12), the progesterone level was its lowest during oestrus. The progesterone level was  $7.99 \pm 2.95$  ng/ml on Day 11 and  $8.04 \pm 2.94$  ng/ml on Day 21 post-mating (Figure 1 and Table). These results were in accordance with the findings of Parakash et al. (7) for routine classification and corpora lutea physiological variations. In pregnant buffaloes milk progesterone concentrations increased during the early luteal phase and stayed elevated on Day 45, the time of the last sample collection. This suggests that the progesterone assay is a valuable diagnostic tool for veterinarians or staff facing difficulties detecting exact oestrus times and asymptomatic oestrus behaviour.

Increased progesterone levels were found in 16 Anatolian buffaloes on Day 19 and Day 24 post-mating; however, 4 of the 16 were determined to be non-

pregnant by rectal palpation. These findings suggest that milk progesterone assay may be used in estimation of early pregnancy in buffaloes as previously reported (3,5) and thus help veterinarians to take the necessary precautions before the next oestrus time if pregnancy does not occur.

Beg and Toteý (13) reported that buffaloes return to oestrus every 19-24 days. Aksoy and Tekin (12) found that the normal oestrus cycle of Anatolian buffaloes was  $21.2 \pm 1.1$  days. In this study, 4 buffaloes, which were later confirmed as non-pregnant, did not return to oestrus at their normal time. Progesterone concentrations in these buffaloes did not decrease on Days 19, 21, and 24. Failure to return to oestrus at normal intervals in buffaloes has been previously reported (3,12,13). Some reproductive problems such as ovarian cysts (2,14) or embryonic mortality (15) may also prevent buffaloes returning to oestrus and may cause false pregnancy detection, especially if they are associated with increased levels of progesterone. Furthermore, cystic ovaries were reported to be an infertility problem in high producing buffaloes (14). In this study, progesterone levels did not decline in 2 of these 4 non-pregnant buffaloes (Figure 2) and luteal structures were palpated on Day 60, but differential diagnosis could not be performed, which could be due to a persistent corpus luteum or a luteinised cyst. In the other 2 non-pregnant buffaloes with elevated progesterone levels, milk progesterone concentration was 1.2 ng/ml on Day 31 and 0.88 ng/ml on Day 45 (Figure 3), and then oestrus symptoms were observed. Embryonic deaths might have occurred in these buffaloes. Thus the milk progesterone test may give an idea and prevent further economic losses by helping to uncover such disorders.

The accuracy rate, sensitivity, specificity, and the detection rate of pregnancy and non-pregnancy for milk progesterone assay in Anatolian buffaloes during Days 19 and 24 were 81.0, 100.0, 56.0, 75.0 and 100.0%, respectively. The pregnancy detection rate of milk progesterone assay in this study was similar to the results reported by Gupta and Prakash (3). In the Gupta and Prakash study (3), the pregnancy estimation rate was 75.0% during Days 22 and 24. Moreover, 5 buffaloes were detected as non-pregnant on Day 19 and Day 24 with milk progesterone assay with a 100.0% detection rate of non-pregnancy (Figure 4). This rate was consistent with the results of Gupta and Prakash (3) and

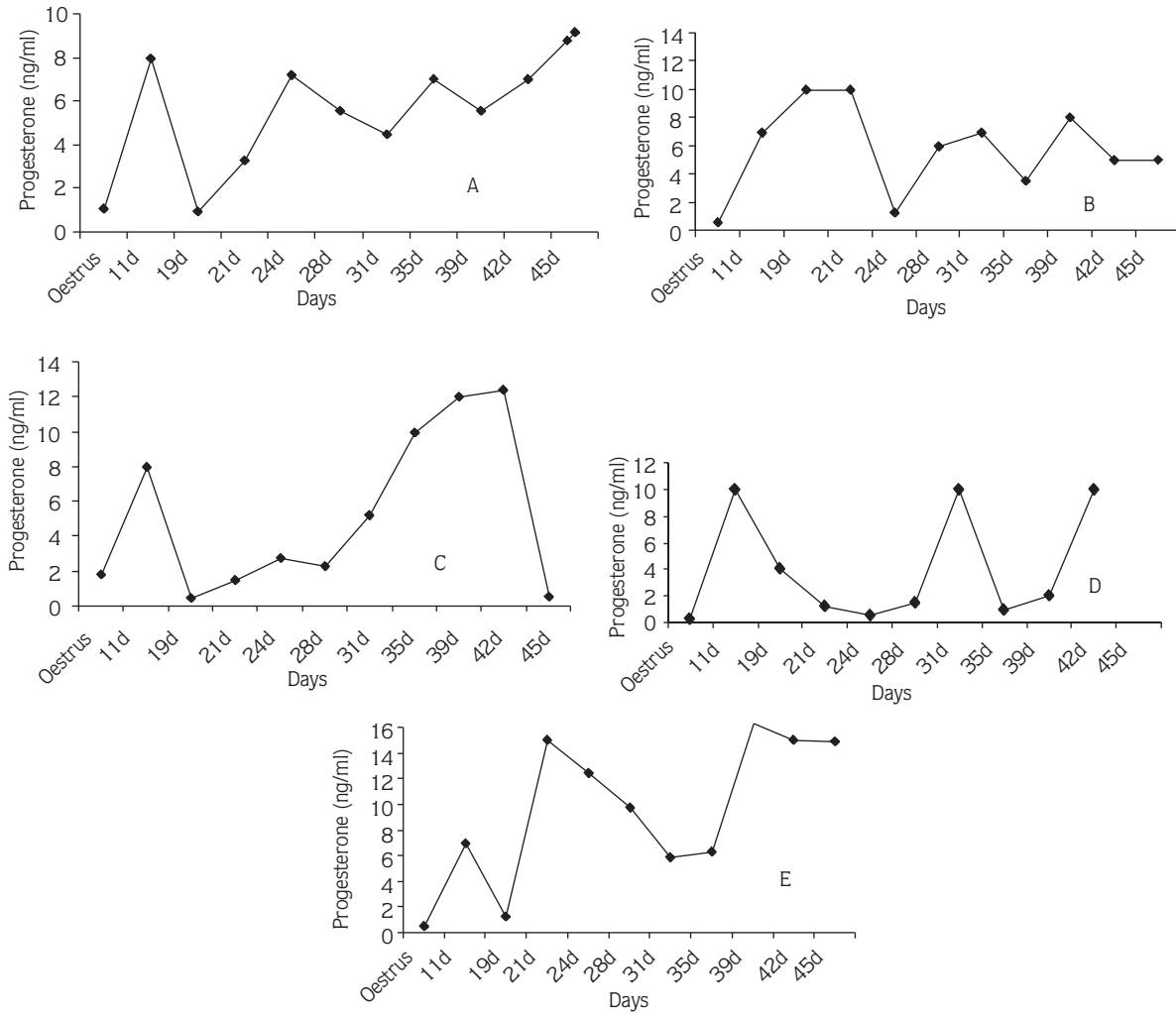


Figure 4. (A,B,C,D,E) Individual progesterone concentrations in buffaloes that returned to oestrus.

Singh and Puthiyandy (16). In addition, progesterone levels decreased ( $0.88 \pm 0.37$  ng/ml) during oestrus in 5 buffaloes in which pregnancy did not occur, and these animals returned to oestrus in the normal time of their cycles (in 19-24 days). On the basis of these determinations, progesterone concentration in Anatolian buffaloes is lower than 1 ng/ml during oestrus (Day 0) and the late luteal phase (Day 19). However, during the early and mid-luteal phase, milk progesterone concentration is approximately 6-8 ng/ml.

In conclusion, milk progesterone assay is a fast and reliable tool to verify oestrus time and to detect non-pregnant buffaloes with a 100.0% accuracy. Thus,

measurement of milk progesterone levels may efficiently be used for detecting buffaloes to be re-submitted to artificial insemination or to be mated. In addition, using this laboratory assay enables farmers and veterinarians to have an idea about early pregnancy and some reproductive failures such as ovarian cysts and embryonic mortality, which is of considerable benefit to the dairy industry.

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