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Estradiol and progesterone levels and expression of estrus behaviour during estrus in repeat breeder cows

A Suresh, C Joseph, T Sarath, R Sureshkumar and S Jaishankar

Abstract
The present study was designed to compare serum estradiol-17β and progesterone levels during estrus of repeat breeders with normally cycling cows and 40 crossbred cows, divided into two groups, normally cycling (Group 1, n=20) and repeat breeders (Group 2, n=20) were used for the study. Blood was collected on the day of estrus for hormonal analyses of estradiol-17β and progesterone using ELISA kits. A significant ($p<0.05$) difference was found in the serum estradiol levels, higher in Group 1 (50.49 pg/ml) than Group 2 (41.85 pg/ml). No significant difference was seen in serum progesterone levels. Lower estradiol-17β concentrations during estrus in repeat breeder cows can affect the final stages of follicular growth and development besides expression of estrus behaviour, and can be a potential cause of repeat breeding.

Keywords: cattle, estradiol-17β, progesterone, repeat breeder

Introduction
Repeat breeders are defined as cyclically normal subfertile animals with no anatomical or infectious regularities, which have failed to conceive with at least three services (Singh *et al.*, 2008) [1]. The incidence of repeat breeding varies from 10-25% across the country (Jayaganthan *et al.*, 2016) [2], and has multiple causes including genetic, nutritional, hormonal, gamete abnormalities, delayed ovulation, luteal insufficiency, infectious or managemental (Selvaraju *et al.*, 2002) [3]. Out of these, hormonal imbalances during various phases of the estrus cycle can be a major, yet hard to detect, cause of repeat breeding.
An improper synchronisation of the hormonal changes during estrus may adversely affect all the upcoming events, from oocyte maturation to embryo development (Gustaffson *et al.*, 1986) [4]. Several studies have indicated endocrine impairment in estrogen (E2), progesterone (P4), or luteinizing hormone (LH) as potential reasons for the repeat breeding phenomenon (Bage *et al.*, 2002, Saumande and Humbolt 2005, Bloch *et al.*, 2006 and Sood *et al.*, 2015) [5, 6, 7, 8]. Therefore, the present study was designed to compare serum estradiol and progesterone levels during estrus of repeat breeders with normally cycling cows and an attempt to explore the differences in estrus behaviour and hormonal levels during estrus as a potential cause of repeat breeding.

Materials and Methods
The present study was conducted at Post Graduate Research Institute in Animal Sciences (PGRIAS), Kattupakkam with forty animals in which 20 normal cycling animals formed Group 1 and 20 repeat breeders formed Group 2. The repeat breeder group consisted of apparently healthy cows that had calved at least once, were less than 10 years of age, inseminated more than three consecutive times with good quality semen but not conceived. All the selected cows were kept under visual observation three times per day at uniform intervals for identification of behavioral estrus signs.

Estrus cycle parameters
The duration and intensity of estrus was recorded in both groups. The intensity of estrus was quantitatively scored using a combination of behavioural and physiological changes as described by Ali (2018) [9]. The scorecard is as follows:

Table 1: Scorecard for the evaluation of estrus intensity

S. No.	Observations	Score	Total score
1.	**Behavioural changes**		
a.	Restlessness and alertness	1	
b.	Mounting	1	
c.	Standing to be mounted	1	
d.	Posture and vocalisation	1	
e.	Chin resting and rubbing	1	
2.	**Physiological changes**		
a.	Vulval oedema		
	Highly oedematous	2	
	Oedematous	1	
	Not oedematous	0	
b.	Frequent urination	1	
c.	Genital discharge		
	Large volume, stringy	2	
	Moderate volume, stringy to mucus	1	
	Sparse volume, viscous	0	
3.	**Gynaecological observations**		
a.	Fern pattern		
	Typical		
	Atypical		
	None		
b.	**Cervical relaxation**		
c.	Uterine tone		
	Highly tonic		
	Tonic		
	No tonicity		

Blood collection

On the day of estrus, 5 ml of blood was collected in non-vacuum blood collection tubes (nVac Tube, Peerless Biotech Pvt Ltd) without anticoagulant for serum separation prior to treatment. The serum was separated by centrifugation for 3000 RPM for 10 minutes and stored at -20°C until hormone analysis.

Prior to the assay all the samples were thawed to room temperature and estradiol-17β and progesterone were estimated using Calbiotech, Inc (CBI) ELISA kits for the respective hormones.

Estradiol 17β estimation

25µl of standards, samples and controls were added to the appropriate wells of a 96-well ELISA plate. 50µl of working solution of estradiol biotin reagent was then added into each well and mixed well with magnetic shaker for 20 seconds and the plate was then incubated at 25 ^{0}C for 45 minutes. After incubation, 100µl of estradiol enzyme reagent were added to all wells and mixed well with magnetic shaker for 20 minutes and the plate was again incubated at 25 ^{0}C for 45 minutes. The wells were then emptied and washed 3 times with 300µl of 1X wash buffer and the plates were blotted with absorbance paper. 100µl of TMB reagent were added into each well and the plate was incubated at 25 ^{0}C for 20 minutes. Finally, 50µl of stop solution was added to each well to stop the reaction and gently mixed for 30 seconds and the Absorbance reading was recorded at 450 nm with microplate ELISA reader within 15 minutes.

Progesterone estimation

20µl of progesterone standards, samples and controls were added to appropriate wells of the 96-well plate. 100µl of working solution of progesterone enzyme conjugate and 50µl of progesterone biotin conjugate were then added to all the wells and incubated for 60 minutes at room temperature. After 60 minutes, the wells were emptied and washed 3 times with 300 ml of 1X wash buffer. The plates were blotted with absorbance paper and once dry, 100µl of TMB substrate were added into each well. The plate was again incubated for 15 minutes, after which 50µl of stop solution was added to each well to stop the reaction. After gentle mixing and shaking of the plates, absorbance reading at 450 nm with microplate ELISA reader within 15 minutes was recorded.

Calculation of results

The mean absorbance value (A_{450}) for each of the respective reference standards, controls and samples was recorded and a standard curve was plotted with the mean absorbance obtained for each reference standards against its concentration in pg/ml for (estradiol-17β) or ng/ml (for progesterone) on a linear graph paper, with absorbance values on the vertical or Y axis, and concentration on the horizontal or X axis. The mean absorbance values for each specimen were used to determine the corresponding concentration of estradiol or progesterone in pg/ml or ng/ml, respectively, from the standard curve.

Statistical analysis

Statistical analysis was performed with the two-tailed independent sample t-test and p values <0.05 were considered statistically significant. All statistical analysis was done using SPSS Statistical software.

Results

A significantly (p<0.01) higher estrus intensity was found in the normally cycling group, when compared to the repeat breeder group (Table 2). However, there was no significant difference in the duration of estrus between the groups.

Table 2: Estrus characteristics (Mean ±SE) of normally cycling and repeat breeder cows

	Duration of estrus (hours)	Intensity of estrus
Group 1	21.45±0.39	11.20±0.39**
Group 2	22.25±0.60	9.05±0.36**

Where, ** indicates p<0.01 level of significance

The mean serum estradiol and progesterone concentrations are represented in Table 3. The serum estradiol value was significantly higher in the normally cycling group than in the repeat breeder group (p<0.05). However, no significant difference was found in serum progesterone levels between the two groups.

Table 3: Mean serum estradiol-17β and progesterone levels during estrus (Mean ±SE)

	Serum Estradiol (pg/ml)	Serum Progesterone (ng/ml)
Group 1	50.49±1.14*	2.41±0.12
Group 2	41.85±3.01*	2.19±0.29

Where, * indicates *p<0.05 level of significance

Discussion

Estradiol-17β has various roles during follicular development and estrus and circulating estradiol concentrations are positively related to the intensity and duration of estrus (Katz *et al.*, 1980; Britt *et al.*, 1986; Lyimo *et al.*, 2000) [10, 11, 12]. While being one of the main factors involved in suppressing Follicular Stimulating Hormone (FSH) concentrations during follicular deviation (Ginther *et al.*, 2000) [13], estradiol has also been shown to induce preovulatory-like FSH and LH

surges in heifers (Kesner *et al.,* 1982) [14]. The estrus intensity, which is directly affected by estradiol levels, is equally important, as the identification of estrus can directly affect pregnancy rates in a herd, particularly in synchronisation and timed artificial insemination protocols (Nogueira *et al.*, 2019) [15].

In the present study, the serum estradiol level was significantly (p<0.05) lower in repeat breeders cows than the normal cycling animals and estrus intensity of the repeat breeder cows also scored significantly (p<0.01) lower than the normally cycling animals indicating the relationship between estrus behaviour and serum estradiol concentrations. However, in this study the duration of estrus was similar in both groups.

The results of this study were lower than those reported by Ahamed *et al.,* (2018) [16], but higher than those of Sood *et al.* (2015) [8] in similarly conducted studies. The latter study also found that the estradiol concentration was higher in repeat breeder than control group, which is contrary to what was found in the present study. The lower level of estradiol in the present study population could account for being at least one cause of repeat breeding as expression of estrus improves fertility and decreases pregnancy losses in lactating dairy cows (Pereira *et al.*, 2016) [17].

Progesterone is secreted by the corpus luteum, which is formed after ovulation. If pregnancy is not achieved, the degeneration of the corpus luteum leads to a decline in progesterone levels by day 18-19, allowing the animal to return to estrus by day 20-22 (Ahammed *et al.,* 2018) [16]. High levels of progesterone during estrus can effectively inhibit estrus behaviour even if high levels of circulating estradiol are present (Allrich, 1994) [18]. Higher plasma progesterone concentrations around estrus can also accelerate the transport of the ova or embryo, which may lead to premature entry of the zygote into the uterus (Newcomb and Rowson, 1975) [19]. Elevated progesterone concentrations during estrus (above 2.5 ng/ml) have been reported as a sign of reproductive failure (Huszenicza *et al.*, 1994) [20].

However, luteal insufficiency (and hence lower progesterone concentrations) has also been reported as a major cause of early embryonic mortality. The present study revealed a lower (although non-significant) level of progesterone during estrus which concurs with Barui *et al.* (2015) [21] who have shown significant differences between progesterone levels of normally cyclic and repeat breeder cows, with lower levels seen in the repeat breeder cattle in their study.

In conclusion, repeat breeding is a multifactorial disorder, in which hormonal imbalances play an important role and lower estradiol concentrations during estrus can affect the expression of estrus behaviour in repeat breeder cattle.

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