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Effect of Systemic Cortisol on Pregnancy Rate in Repeat Breeding Cows during Early Pregnancy

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ABSTRACT

Background: The effect of systemic cortisol on pregnancy rate during early pregnancy in repeat breeding cows was estimated.

Methods: Oestrus synchronisation was done in 20 repeat breeders and samples were collected on different days of post-insemination to estimate cortisol.

Result: Trans-rectal ultrasonography on 26th day of post insemination revealed a pregnancy rate of 45%. When two groups were compared, serum, salivary and urinary cortisol level of non-pregnant animals were significantly ($P < 0.05$) increased than that of pregnant animals on different days of post-insemination. Within non-pregnant animals, serum, salivary and urinary cortisol levels showed a significant ($P < 0.05$) variation between different days of post insemination, but this variation was not observed in pregnant animals. Spearman rho correlation revealed positive association ($P < 0.05$) of systemic cortisol with pregnancy rate. In non-pregnant animals, salivary and urinary cortisol levels were observed to be positively correlated ($P < 0.05$) with serum cortisol. The results indicate that systemic cortisol has influence on pregnancy rate in repeat breeding cows, this may be due to its effect on embryo implantation and hormonal balance, which requires further validation. Association of salivary and urinary cortisol with serum cortisol indicates their use as non-invasive samples to monitor the hypothalamic-pituitary-adrenal axis activity in repeat breeders.

Key words: Cortisol, Conception rate, Implantation, Repeat breeder cow.

INTRODUCTION

Reproductive inefficiency of cattle due to repeat breeding syndrome is an expensive hitch in profitable dairy production, as the age at first calving in heifers is delayed and the inter-calving period is extended, thus leading to lowering of calf crop (Thakur *et al.,* 2006). Repeat breeder cattle were defined as sub-fertile animals with apparantely normal genitalia but failed to conceive with at least three consecutive services Yusuf *et al.,* 2010). Incidence of repeat breeding may vary among herds from 9% to 24% (Bulman and Lamming 1978; Bartlett *et al*., 1986; Waldmann *et al*., 2001). Optimal treatment strategy for repeat breeding remains elusive because of its multifactorial etiology, which has been attributed to inappropriate time of insemination (Hunter and Greve, 1997), abnormalities of the reproductive organs (Kubar and Jalakas, 2002) and hormonal disorders (Waldmann *et al*., 2001). Nevertheless, clinically normal genital organs (Stroud *et al.,* 1991) and uterine environment of repeat breeders have been found to be suitable for the maintenance of pregnancy (Tanabe *et al.,* 1985).

Pregnancy failure in repeat breeding cows has also been associated with embryonic mortality (Linares, 1981, Gustafsson and Larsson, 1985; Hawk *et al.,* 1955). It has been postulated that healthy sub-fertile cows may have impairments in maturation of follicles (Stroud *et al.,* 1991), follicular growth (Maurer and Echternkamp, 1985), or oocyte development (Boland *et al.,* 2001; McEvoy *et al.,* 2001). Nutrition-dependent metabolic and endocrine status of cows were related to normal development of oocytes (Graham *et al.,* 1995; Hazeleger *et al.,* 1995; Driancourt and Thuel, 1998).

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Several studies have indicated the endocrine impairment in progesterone, estrogen and luteinizing hormone levels as putative cause for the repeat breeding in cows (Bage *et al.,* 2002, Saumande and Humbolt, 2005, Bloch *et al.,* 2006). Sustained adrenal stimulation associated with environmental or social stress could be one of the factors that contribute to these hormonal imbalances in repeat breeders (Bage *et al.,* 2000).

Under stressful condition, activation of hypothalamic-pituitary-adrenal (HPA) axis leads to increased secretion of corticotropin releasing factor, adrenocorticotropic hormone and cortisol (Lucassen *et al.,* 2014) therefore, measurement of cortisol is generally considered an indicator for stress

(Dallman *et al.,* 1987, Sapolsky *et al.,* 2000). Various factors can influence the circulating level of cortisol in dairy cattle like circadian rhythmicity (Lefcourt *et al.,* 1993), environmental cues like cold, heat, humidity and wind (Chen *et al.,* 2005). To reduce the confounding effect of invasive procedure on cortisol level, various studies have validated the association of salivary (Perez *et al.,* 2004), milk (Sgorlon *et al.,* 2015), hair (Comin *et al.,* 2011), faeces (Morrow *et al.,* 2002) and urine (Redbo, 1993) cortisol levels with stress in dairy animals. Previous studies reported the impact of adrenocorticotropic hormone or cortisol on the reproductive performance during oestrus in repeat breeders (Bage *et al.,* 2000; Widayati *et al.,* 2019). However, the effect of endogenous cortisol on pregnancy rate during early stage of pregnancy in repeat breeders has not been studied yet. Thus, the objective of the present study was to investigate the effect of systemic cortisol on pregnancy rate of repeat breeders during early pregnancy stage.

MATERIALS AND METHODS

Experimental animals

The study was conducted during winter (from mid-December 2018 to mid-March 2019) to avoid heat stress in an organised private dairy cattle farm. The average temperature during the period of study was 29°C. All the selected animals were normal cyclic, had normal estrus duration and with no previous history of dystocia, metritis, retained placenta. A total of 76 Jersey crossbred animals were screened to identify the true repeat breeding cows for inclusion in present study (study population). Based on examination and definition of repeat breeding cattle twenty cows (days in milk – 290 ± 30) were considered as repeat breeders. In the selected animals, average number of artificial inseminations done was 6.0 ± 1.8 (range: 4 – 9). Genital tract health of all the selected cows was reaffirmed by two successive trans-rectal ultrasound examinations with Sonoscape S2 V 7.5-MHz linear array transducer (SonoScape Medical Corp.) separated by 10 days interval. All the animals were housed in the covered loose pen with an adjacent outside yard and they were fed according to NRC (2001) recommendations. Every cow was milked twice a day (06.30 h and 17.30 h) in a tandem parlour. Body condition score (BCS) was determined in all cows before initiation of synchronization protocol according to Edmondson *et al*. (1989) and body condition score of selected cows falls within the range of 2.5 – 3.5. All the animals were dewormed with fenbendazole (Fendikind, Mankind Pharma Vet) at 7.5mg/kg body weight and they were also supplemented with standard mineral mixture (Agrimin® Forte, Virbac Animal Health India Private Limited) at the rate of 30 mg/kg body weight for one month.

Experimental protocol

Oestrus synchronization and blood collection protocol is represented in fig 1. After selection, all the cows were synchronized using the Ovsynch protocol. The 10 µg of GnRH analogue - Buserelin acetate (Gynarich®, Intas Pharmaceuticals Limited, India) was given on 0th and 9th day and 500 µg of $PGF_{2\alpha}$ synthetic analogue-Cloprostenol sodium (Pragma®, Intas Pharmaceuticals Limited, India) injected on 7th day. All the animals were inseminated 16 and 24 hrs after 2nd GnRH injection with crossbred Jersey frozen French mini semen straws (Nucleus Jersey and Stud Farm, Ooty, India). Blood, saliva and urine samples were collected before milking and feeding in early morning from all the cows on day 14, 18, 20, 22 and 25 post-insemination. On 26th day of post insemination all the selected animals were screened for pregnancy by trans-rectal ultrasound examinations with Sonoscape S2 V 7.5-MHz linear array transducer (SonoScape Medical Corp.).

Estimation of serum cortisol

The blood samples were collected before milking and feeding in the morning from all the cows by jugular vein puncture in a clot activator tube (Quantum Biomedicals, India). The serum was separated by centrifugation at 300 g for 20 minutes and stored at -20°C for further estimation. Serum cortisol was determined with a solid phase enzyme immunoassay (DetectX® Cortisol enzyme immunoassay kit, Arbor Assays Inc., USA). The final absorbance of samples was measured in the Epoch Microplate Spectrophotometer (BioTek Instruments, Inc., USA) at 450 nm.

Estimation of salivary cortisol

Saliva was collected according to Negrao *et al*. (2004) by swabbing technique with sight modification. A synthetic cotton swab attached to a plastic syringe was rubbed up and down in the cheeks and over the tongue and then piston was depressed to collect 2 ml of saliva, which was immediately kept in – 20 °C until analyses. Salivary cortisol was determined with a solid phase enzyme immunoassay (DetectX® Cortisol enzyme immunoassay kit, Arbor Assays

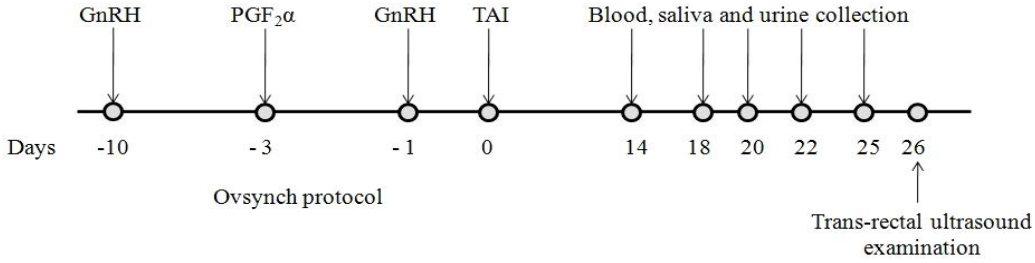

Fig 1: Schematic of oestrus synchronization and blood collection protocol of experiment.

Inc., USA). The final absorbance of samples was measured in the Epoch Microplate Spectrophotometer (BioTek Instruments, Inc., USA) at 450 nm.

Estimation of urinary cortisol

Urine sample was collected according to Higashiyama *et al*. (2005) in the mid-stream during spontaneous urination and filtered to remove the debris and stored at - 20°C until analyses. Urinary cortisol was determined with a solid phase enzyme immunoassay (DetectX® Cortisol enzyme immunoassay kit, Arbor Assays Inc., USA). The final absorbance of samples was measured in the Epoch Microplate Spectrophotometer (BioTek Instruments, Inc., USA) at 450 nm.

Statistics

Data were expressed as standard error of mean. Shapiro-Wilk test was used to assess the normal distribution of data. Analysis of variance (ANOVA) with Bonferroni correction was applied to compare the serum, salivary and urinary cortisol levels on different days of post-insemination between groups. Repeated measure ANOVA with Bonferroni correction was used to determine the significant variation in serum, salivary and urinary cortisol level among different post-insemination days within each group. Pearson's correlation was performed to assess the association of salivary and urinary cortisol with serum cortisol. Sperman rho correlation was used to determine the association serum, salivary and urinary cortisol levels with pregnancy rate. $P < 0.05$ was considered as statistically significant. All analyses were performed using SPSS IBM Version 23 software.

RESULTS AND DISCUSSION

Trans-rectal ultrasound examination of all the twenty inseminated repeat breeders on 26th day post-insemination revealed the presence of amniotic vesicle in 9 (45%) cows and non-pregnancy in 11 (55%) cows. The finding of the present study is similar to the previous reports on use of ovsynch protocol to improve pregnancy rate in repeat breeders (Fricke *et al*., 2003; Caraba and Velicevici, 2013; Jayaganthan *et al.,* 2016).

Days of the animals in milk for pregnant and non-pregnant animals were 236 ± 24 and 337 ± 50 respectively. On comparing the serum cortisol level of two groups, significant increase was observed on 18th ($P < 0.0001$), 20th ($P < 0.0001$), 22nd ($P = 0.0004$) and 25th ($P = 0.0003$) days of post-insemination in non-pregnant animals (Fig 2a). Significant increase in salivary cortisol was observed on 18th ($P < 0.0001$), 20th ($P < 0.0001$), 22nd ($P < 0.0001$) and 25th ($P = 0.0130$) days of post-insemination in non-pregnant animals when compared with pregnant animals (Fig 2b). On comparing with pregnant animals, urinary cortisol level of non-pregnant animals was significantly increased on 18th ($P < 0.0001$) and 20th ($P = 0.0008$) days of post-insemination (Fig 2c). These significant increase in serum, salivary and urinary cortisol level of non-pregnant animals compared to that of pregnant animals, indicates the association of cortisol and early pregnancy complications (Thatcher *et al.,* 1984; Schafer-Somi, 2003). Previous studies in repeat-breeding cows on early embryonic mortality have also recorded loss of embryos in the range from 8% to 30% (Boyd *et al.,* 1969; Hawk, 1979; Ayalon, 1978; Thatcher *et al.,* 1994) with most

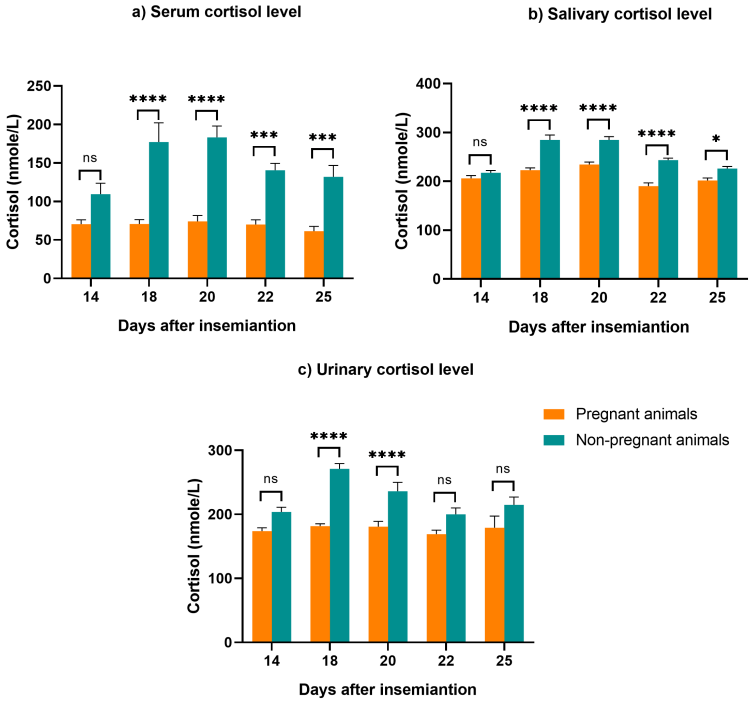

Fig 2: Changes in serum, salivary and urinary cortisol level in different days of post-insemination in pregnant (n = 9) and non-pregnant (n = 11) animals. (**** - differs significantly at $P < 0.0001$; *** - differs significantly at $P < 0.001$; * - differs significantly at $P < 0.05$; ^{ns} - no significant difference; Mean and SE).

embryonic losses occurring between 8 and 16 days of breeding (Roche *et al.,* 1981). Initial trophoblast-decidua interactions occur through molecular dialogue initiated during blastocyst attachment by signalling molecules like integrins and fibronectin (Burrows *et al.,* 1996) but physiological concentration of glucocorticoids can suppress the expression of these signalling molecules (Ryu *et al.,* 1999). Furthermore, successful implantation requires co-ordinated role of pro-inflammatory cytokines, such as interferones, interleukins-1,2,4,6,10, granulocyte macrophage -colony stimulating factor, tumor necrosis factor-α (Schafer-Somi, 2003; Raheem, 2018) but glucocorticoids were known to impair this cytokine signalling cascade required for implantation (Michael and Papageorghiou, 2008).

Comparing serum cortisol of non-pregnant animals with different post-insemination days, revealed a significant increase on 18th and 20th days than 14th (18th day: P = 0.0157; 20th day: P = 0.0273) and 22nd (20th day: P = 0.0253) days (Fig 3b). However, no significant difference was observed in the serum cortisol of pregnant animals among different post-insemination days (Fig 3a).

Within group comparison of salivary cortisol in non-pregnant animals revealed significant increase on 18th and 20th days than 14th (18th day: P = 0.0001; 20th day: P = 0.0009), 22nd (18th day: P = 0.0033; 20th day: P = 0.0089) and 25th (18th day: P = 0.0013; 20th day: P = 0.0022) days (Fig. 4b). No significant change was seen in salivary cortisol of pregnant animals on different post-insemination days (Fig. 4a). Among different post-insemination days the urinary cortisol level of non-pregnant animals showed a significant increase on 18th and 20th days than 14th (18th day: P = 0.0016; 20th day: P = 0.0143) and 22nd (18th day: P = 0.0027; 20th day: P = 0.0125) days. (Fig 5b). Urinary cortisol level of pregnant animals did not differ significantly on different days of post-insemination (Fig 5a).

Significant variation in the cortisol level among different post-insemination day was observed only in non-pregnant animals. Reports of previous studies suggests that cortisol is an important factor in inducing the pulsatile release of PGF2α from the inter caruncular region of deciduas, which causes the regression of corpus luteum and reduces the circulating level of progesterone needed for pregnancy maintenance (Ginther *et al*., 2009; Duong *et al*., 2012; Duong *et al*., 2012a). Cortisol can also indirectly suppress the progesterone level by reducing basal level of luteinizing hormone as well as luteinizing hormone – releasing hormone

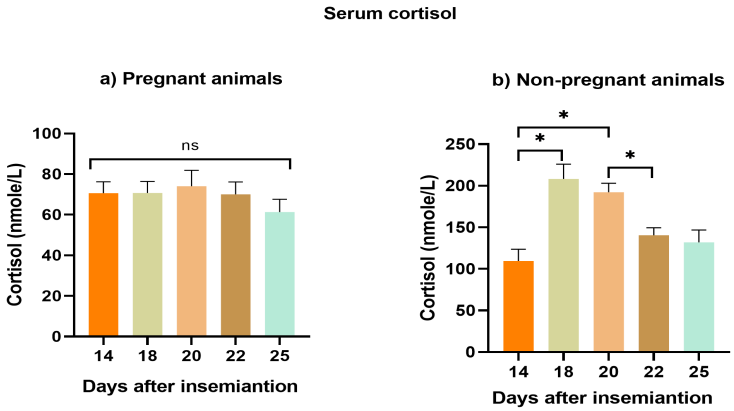

Fig 3: Within group variation of serum cortisol among different post-insemination days in pregnant (n = 9) and non-pregnant (n = 11) animals. (* - differs significantly at P < 0.05; ^{ns} - no significant difference; Mean and SE).

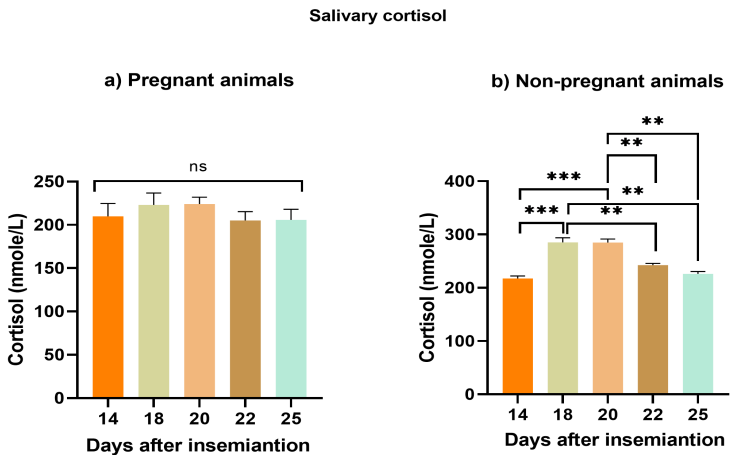

Fig 4: Within group variation of salivary cortisol among different post-insemination days in pregnant (n = 9) and non-pregnant (n = 11) animals. (*** - differs significantly at P < 0.001; ** - differs significantly at P < 0.01; ^{ns} - no significant difference; Mean and SE).

induced release of luteinizing hormone from pituitary cells of bovine (Padmanabhan *et al.,* 1983).

In the current study, salivary and urinary cortisol levels of non- pregnant animals were found to be positively correlated with serum cortisol in all the days of study (Table 1), which indicates salivary and urinary cortisol can be used as an indicator for measuring the hypothalamic-pituitary-adrenal axis activity in repeat breeding cows. Plasma cortisol has been routinely used as an indicator of stress in animals (Broom and Johnston, 1993) but it provides only single point-in-time estimate and also restraining of animals during blood collection may interact with plasma cortisol level (Willett and Erb, 1972; Alam and Dobson, 1986). Plasma cortisol measures the total cortisol which includes both corticosteroid-binding globulin bound cortisol and free cortisol (Coolens *et al.,* 1987). Alternatively, salivary and urinary samples can be used to estimate free cortisol. Salivary cortisol concentration was unaffected by salivary flow rate because cortisol enters saliva by passive diffusion (Riad-Fahmy *et al.*, 1982) and also it was highly correlated to changes in serum cortisol level (Vining *et al.,* 1983). Urinary cortisol reflects systemic cortisol concentration that enters the urine *via* glomerular filtration (Biesel *et al.,* 1964) and also it has the merit of providing an integrated index of cortisol secretion over the period preceding sampling (Lasley, 1985). These non-invasive cortisol levels observed in the present study correlate with serum cortisol in various species including horses (Peeters *et al.,* 2011), cattle (Perez *et al.,* 2004), rats (Thanos *et al*., 2009) and humans (Hellhammer *et al.,* 2009).

Serum cortisol level was positively associated with pregnancy rate on day 18th ($P < 0.0001$), 20th ($P < 0.0001$), 22nd ($P < 0.0001$) and 25th ($P < 0.001$) days of post insemination and salivary cortisol level was positively associated with pregnancy rate on day 18th ($P < 0.0001$), 20th ($P < 0.0001$), 22nd ($P < 0.001$) and 25th ($P < 0.001$) days of post insemination. Urinary cortisol level was positively associated with the pregnancy rate on 18th ($P < 0.0001$), 20th ($P < 0.05$) and 22nd ($P < 0.05$) days of post insemination (Table 2). This indicates that the systemic cortisol level may be used to predict the glucocorticoid induced early pregnancy loss in repeat breeders. Previous studies have reported that in repeat breeders the developmental ability of embryo was compromised (Linares, 1981, Gustafsson and Larsson, 1985; Albihn *et al*., 1989), as cortisol can induce the luteolysis and reduce the circulating level of progesterone (Spicer and Chamberlain 1998). Depending upon the physiological status, cortisol can alter the embryo implantation and pregnancy rate in heifers (Duong *et al.,* 2012). Elevation of cortisol concentration is associated with induction of oocyte apoptosis and deteriorates oocyte quality (Prasad *et al.,* 2016), percentage of abnormal oocytes in repeat breeders may range up to 52.5% (Kurykin *et al.,* 2011). Chronic and acute stress can affect pulsatile release reproductive hormones from hypothalamic-pituitary-gonadal

Table 1: Correlation of serum cortisol with salivary and urinary cortisol on different days after insemination in non-pregnant repeat breeders.

Pearson's n correlatio	Serum cortisol			
	Salivary cortisol		Urinary cortisol	
	R value	P value	R value	P value
Days 14	0.9132	0.0006	0.9112	0.0016
Day 18	0.9146	0.0015	0.9637	0.0001
Day 20	0.9154	0.0005	0.8772	0.0042
Day 22	0.9305	0.0024	0.9800	0.0001
Day 25	0.9131	0.0006	0.8464	0.0080

Table 2: Correlation of pregnancy rate with salivary and urinary cortisol on different days after insemination.

Spearman correlation	Pregnancy rate		
	Serum cortisol	Salivary cortisol	Urinary cortisol
	R value	R value	R value
Days 14	0.3851^{ns}	0.2502 ^{ns}	0.6508 ^{ns}
Day 18	0.8664****	0.8189****	0.8666****
Day 20	0.8660****	0.8674****	0.5540 *
Day 22	0.8660****	0.8660***	0.6099 *
Day 25	0.7124***	0.7698***	0.4067 ^{ns}

(**** - differs significantly at $P < 0.0001$; *** - differs significantly at $P < 0.001$; ^{ns} - no significant difference).

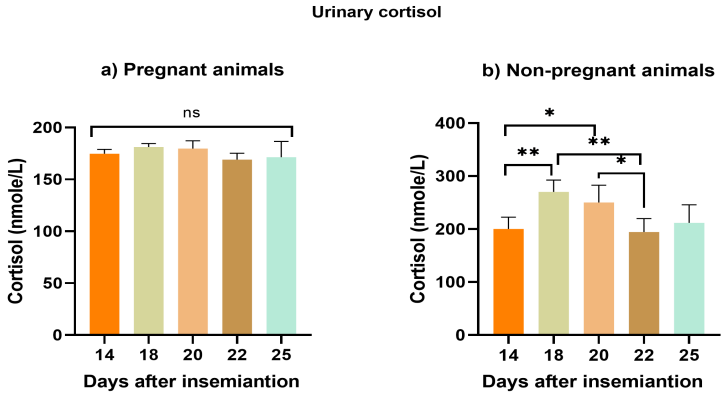

Fig 5: Within group variation of urinary cortisol among different post-insemination days in pregnant (n = 9) and non-pregnant (n = 11) animals. (** - differs significantly at $P < 0.01$; * - differs significantly at $P < 0.05$; ^{ns} - no significant difference; Mean and SE).

axis and reduces the reproductive performance of animals (Walker *et al.,* 2008; Dobson *et al.,* 2003; Smith *et al.,* 2003). Hence, cortisol can be used as a biomarker for assessing the stress induced early pregnancy changes in repeat breeders.

CONCLUSION

Association of systemic cortisol level with the pregnancy rate during early pregnancy in repeat breeders indicates that the systemic cortisol level may have effect on embryo implantation and hormonal balance in these animals, which requires further validation. Changes in concentration of salivary and urinary cortisol during early pregnancy of repeat breeders were similar to serum cortisol concentration and thus they can be used as a non-invasive technique to measure the hypothalamic-pituitary-adrenal axis activity in repeat breeding cows.

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