



Efficiency of Heatsynch Protocol in Estrous Synchronization, Ovulation and Conception of Dairy Buffaloes (*Bubalus bubalis*)

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ABSTRACT : The objectives of this study were to test the efficacy of induction of estrus and determine the timing of ovulation in relation to preovulatory LH and estrogen surges in cycling Murrah buffaloes subjected to Heatsynch protocol (GnRH-PGF_{2α}-Estradiol benzoate). In experiment 1, the buffaloes (n = 10) were treated with Heatsynch protocol and observed for estrus and ovulation. In experiment 2 and 3, 30 cycling Murrah buffaloes were used to investigate the efficacy of Heatsynch protocol in terms of conception rates in summer (experiment 2) and winter (experiment 3) seasons. Fixed time A.I. was performed in all the buffaloes at 48 and 60 h post-estradiol benzoate (EB) injection. All buffaloes responded to the Heatsynch protocol with expression of estrus for which ovulations were induced in 8 buffaloes (80%). Mean time interval from the EB injection to ovulation was 50.0±2.0 h (range 44.0 to 60.0 h). The interval from the end of LH surge to ovulation was 18.5±2.47 h (range 8 to 26 h). The interval from end of estrogen surge to ovulation was 26.75 ±2.07 h (range 22 to 36 h). Mean LH peak after EB injection occurred at 20.81±1.61 h (range 14 to 28 h) and mean estrogen peak after EB injection occurred at 9.62±1.03 h (range 7 to 16 h). Hence, the mean estrogen peak preceded the mean LH peak by 11 h. It was observed that the percentage of conceptions to total number of estruses for control buffaloes was 18 and 30 in summer and winter, respectively, whereas it increased to 26 and 40 in Heatsynch treated buffaloes in respective seasons. The results suggest the possibility of using Heatsynch treatment followed by fixed time A.I. in buffaloes for fertility improvement, especially since the incidence of silent heat in buffaloes is very high. (**Key Words :** Buffalo, Heatsynch, Estrus Synchronization, Timed Artificial Insemination, Ovulation)

INTRODUCTION

Buffaloes play a prominent role in rural livestock production in Asia and India in particular. Silent estrus is perhaps the most important factor leading to poor reproductive efficiency in buffaloes (Kanai and Shimizu, 1983; Prakash et al., 2002; Madan and Prakash, 2007) especially during hot summer months. As the signs of estrus in buffaloes are less obvious than in cattle, the estrus detection accuracy is one of the major problems limiting the use of A.I. in this species. Various estrus synchronization protocols have been tried (Singh et al., 2000) among many other reproductive technologies for improving the fertility of buffaloes. A novel synchronization protocol named Ovsynch was developed (Pursley, 1995) in cows, which requires a three injection schedule (GnRH-PGF_{2α}-GnRH) for synchronization of ovulation. The technique was

successfully carried out in cycling buffaloes (Paul and Prakash, 2005) for synchronization of ovulation and fixed timed AI. Very recently an estrus synchronization protocol called Heatsynch in cattle has been developed (Pancarci et al., 2002) which makes use of a combination of GnRH-PGF_{2α}-Estradiol cypionate injection. Barros et al. (2000) and Fernandes et al. (2001) had also successfully tested the similar protocol using estradiol benzoate in place of estradiol cypionate. Estradiol benzoate is a less expensive hormone in place of second GnRH injection of Ovsynch protocol. The major advantages of heatsynch are reduced hormone costs and somewhat easier scheduling and implementation, since all injections and A.I. are at 24 and 48 h interval in cows. Heatsynch protocol has not been attempted in buffaloes. Further, no report is available on evaluating the interrelationships of hormones (progesterone, estradiol and LH) during the critical periovulatory period in Heatsynch protocol even in cows.

Keeping the above information in view, we conducted this investigation to i) determine the timing of the preovulatory estrogen and LH surge to ovulation following Heatsynch, and ii) compare conception rates following

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timed AI (TAI) after Heatsynch to those achieved with conventional AI after detection of estrus in summer and winter seasons.

MATERIALS AND METHODS

Experimental animals

Forty five cycling Murrah buffaloes (2nd-5th parity) were selected from NDRI (National Dairy Research Institute) herd for various sets of experiments. The animals selected for the study were free from any anatomical and reproductive disorders and were not suffering from any health problems. All the animals were kept under loose housing system in the experimental animal paddock and milking paddocks. During the experimental period, the environmental temperature fluctuated between a minimum of 1.1°C and a maximum of 39.0°C. The animals were fed as per standard feeding practices and guidelines employed for buffaloes.

Experiment 1: Endocrine changes and timing of ovulation

The experiment was carried out to determine the timing of ovulation and changes in peripheral plasma concentrations of progesterone, estrogen and LH in Murrah buffaloes (n = 10) treated with Heatsynch protocol whereas (n = 5) were non-cycling on the basis of progesterone analysis therefore not taken for experiment. Ovulation was induced and synchronized using the Heatsynch protocol as described by Pancarci et al. (2002). It consisted of an injection of the GnRH analogue (Receptal[®] VET, Intervet India Pvt. Ltd. Pune, India; 10 µg i.m.) followed 7 days later by an analogue of PGF_{2α} (Lutalyse; Pharmacia and Upjohn, Puurs, Belgium; 25 mg i.m.) and then followed 24 h later by estradiol benzoate (EB; Sigma, USA; 1 mg dissolved in absolute alcohol; i.m.). Beginning on the day of EB administration, the buffaloes were monitored for signs of behavioral estrus by visual detection and parading vasectomized buffalo bull every 6 h for 30 min. Uterine tone was monitored by trans rectal palpation of uterus. The timing of ovulation was determined by palpation of ovarian structure at 2 h intervals beginning from the initial signs of estrus until ovulation was confirmed by disappearance of a large follicle on one of the ovaries. Blood samples (5 ml) were collected by jugular veinpuncture at 2 h intervals starting from the time of EB injection until 6 h after occurrence of ovulation. Blood samples were collected in EDTA coated tubes, cooled on ice and then centrifuged at 4°C. The plasma was frozen and stored in 3 aliquots at -20°C until assay for LH, estrogen and progesterone.

Parameters recorded were: (i) behavioral and secondary signs of estrus, (ii) periovulatory LH, total estrogen and progesterone profile and (iii) timing of ovulation in relation

to EB treatment and to the preovulatory estrogen and LH surges.

Experiment 2 and 3: Conception rates to Heatsynch protocol and A.I. in summer and winter seasons

This experiment was conducted to compare conception rates to TAI in Murrah buffaloes synchronized with the Heatsynch protocol, with those bred after detection of spontaneous estrus (control). Fifteen cycling Murrah buffaloes were treated with Heatsynch protocol (as in Experiment 1) and TAI was performed at 48 and 60 h after the EB treatment in summer season (May to Aug.; Experiment 2). Another fifteen cycling Murrah buffaloes were treated with Heatsynch protocol (as in Experiment 1) in winter season (Nov. to Jan.) and TAI was performed at 48 and 60 h after the EB treatment. The timing of insemination was based on the timing of ovulation recorded in buffaloes under experiment 1. Untreated buffaloes belonging to the farm which were under identical feeding regimen served as control; among them 36 Murrah buffaloes that had been observed in spontaneous estrus in summer season and 78 buffaloes that had been observed in spontaneous estrus in winter season during the corresponding periods in Heatsynch treated buffaloes were inseminated as a matter of routine, 12 and 24 h after detection of estrus. Pregnancy diagnosis post-insemination was determined by transrectal palpation 60 days post-AI in animals which had not returned to estrus.

Hormone assay

Enzymeimmunoassay (EIA) for LH: Chemicals: The chemicals purchased from M/s Sigma Chemical Company, St. Louis, USA were biotinamidocaproate-N-hydroxysuccinimideester (Biotin), dimethylsulfoxide (DMSO), bovine serum albumin (BSA), polyoxyethylene-sorbitan monolaurate (Tween 20), streptavidin - peroxidase, ureum peroxide, 3,3',5,5'-tetramethyl benzidine (TMB), N,N-dimethyl-formamide, isobutylchloroformate and thiomersal. The chemicals used in preparation of various buffers were purchased locally from the reputed farms.

The concentrations of LH were determined by EIA using 20 µl of plasma in a biotin-streptavidin amplification system and a second antibody coating technique previously validated for buffalo plasma (Prakash et al., 2002). The LH standard curve ranged from 6.25 to 800 pg/20 µl which corresponded to 0.31-40 ng/ml LH. The sensitivity of the assay for LH in buffalo plasma was 6.25 pg/20 µl, which corresponded to 0.31 ng/ml plasma. The intra assay and inter assay coefficients of variation of LH were found to be 6.4 and 12.3 percent respectively. The bovine LH antiserum (USDA-309-684P) was highly specific for LH (USDA-bLH-B-6). The cross-reactivity of the bLH antisera (USDA-

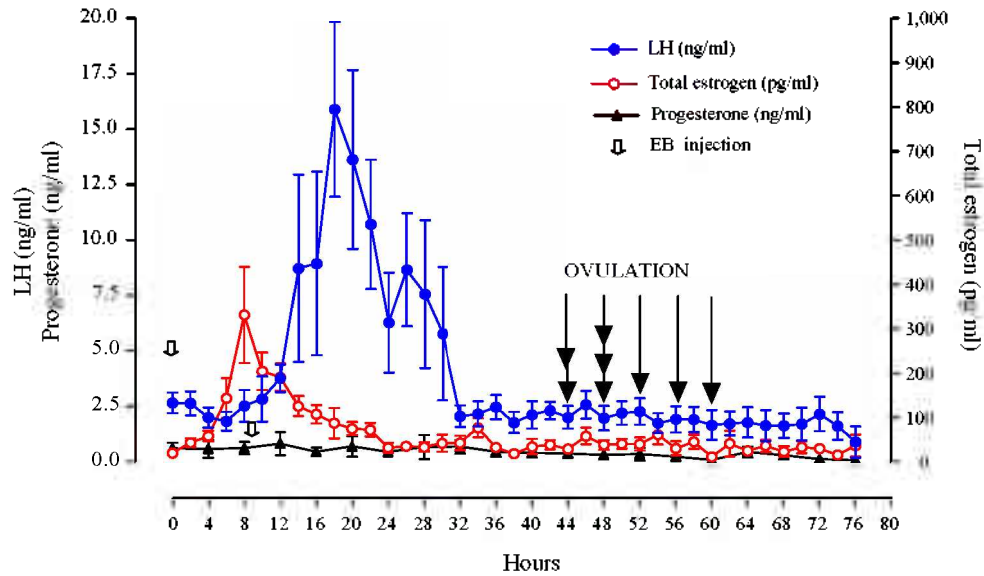


Figure 1. Changes in plasma LH, total estrogen and progesterone (Mean \pm SEM) profile after administration of estradiol benzoate in responder buffaloes (n = 8) treated with Heatsynch protocol.

309-684P) with USDA-bFSH-B-1, USDA-bTSH-I-1, USDA-bGH-B-1, and USDA-bPRL-B-1 was less than 0.7% (data provided by the USDA).

Enzymeimmunoassay (EIA) for estrogen : Highly sensitive heterologous EIA procedure for estrogen estimation in buffalo plasma using the second antibody coating technique was followed (Mondal et al., 2006). The procedure employed 50 μ l of extracted and reconstituted plasma samples, antiserum against estradiol 17 β -17-HS-BSA. This procedure used estradiol 17 β -horse radish peroxidase as the enzyme conjugate. The sensitivity of the assay in extracted plasma was 0.2 pg/50 μ l/well which corresponds to 1.45 pg/ml of plasma. The intra- and interassay coefficients of variation of the assays were 6.3 and 9.5 for total estrogen.

Radioimmunoassay for progesterone : Progesterone was estimated by simple, direct radioimmunoassay following a previously validated procedure (Kamboj and Prakash, 1993). Radioimmunoassay of progesterone estimation was carried out in plasma using 20 μ l of plasma. The sensitivity of the assay for progesterone was 4 pg/tube, which corresponds to 0.2 ng/ml. The intra and inter-assay coefficients of variation for progesterone were 8.4 and 12.0 percent, respectively.

Statistical analysis

Mean and standard error were calculated by using the Graph pad Prism software package-1995. Changes in plasma progesterone concentrations during treatment and plasma LH and estrogen concentrations after the EB administration were analyzed using ANOVA for repeated measures in time. A Chi-square test was used to compare pregnancy rates to TAI versus insemination at spontaneous

estrus in summer and winter season.

RESULTS

Experiment 1: Efficacy of Heatsynch treatment

Estrus synchronization response in terms of ovulations was 80% i.e 8 out of 10 treated buffaloes had ovulated.

Estrus behavior : All the 10 buffaloes treated with Heatsynch protocol exhibited signs of estrus. The major estrus symptoms expressed were swollen vulva, excitement, chasing by bull, frequent urination and uterine tone. Minor estrus symptoms expressed were bellowing, bull mounting, mucus discharge, chin resting on other animals and tail raising. In general all the signs of estrus exhibited were prominent with higher intensity ($p < 0.05$) in comparison to those observed after onset of spontaneous estrus.

Endocrine changes : The temporal changes in plasma LH, estrogen and progesterone in plasma after estradiol benzoate injection are presented in Figure 1.

Changes in plasma LH levels post estradiol benzoate administration : The peak LH concentrations recorded after estradiol benzoate injection was 15.88 ± 3.94 ng/ml (range 4.47 to 33.12 ng/ml; Table 1). The LH levels stayed low prior to the onset of LH surge and after the end of LH surge until ovulation in individual buffaloes. The mean duration of LH surge was 20.75 ± 1.99 h with the range of 16.0 to 32.0 h (Table 1). LH peak occurred 20.81 ± 1.61 h after estradiol benzoate injection ranging from 14.0 to 28.0 h among individual buffaloes. Graphical depiction (Figure 1) of the overall mean LH concentration calculated from the pooled data of all eight animals over a time scale taking the time of estradiol benzoate injection as 0 h showed that the

Table 1. Chronological order of events occurring in buffaloes (n = 8) subjected to Heatsynch protocol

Parameters	Animals	Mean±SEM	Range
LH peak concentration (ng/ml)	8*	15.88±3.94	(4.47-33.12)
Duration of LH surge (h)	8*	20.75±1.99	(16-32)
Timing of events (h)			
a) Onset of estrus after EB injection	8*	16.75±1.25	(12-24)
b) LH peak after EB injection	8*	20.81±1.61	(14-28)
c) Onset of estrus to ovulation	8*	33.25±1.60	(26-42)
d) Ovulation after EB injection	8*	50.00±2.00	(44-60)
e) Ovulation after PGF _{2α} injection	8*	74.00±2.00	(68-84)
f) Ovulation after end of LH surge	8*	18.50±2.47	(8-26)

Out of 10 treated buffaloes, ovulatory response was recorded in 8 animals.

mean LH concentration increased steeply from 2.66±0.45 ng/ml at the time of estradiol benzoate injection to peak value of 15.88±3.94 ng/ml at 18 h, dropping sharply thereafter to basal levels of 2.06±0.48 ng/ml at about 32 h. No LH surge was recorded in non responder buffaloes (Figure 2).

Changes in plasma estrogen levels post estradiol benzoate administration : The mean peak of estrogen concentrations recorded for buffaloes was 330.91±108.37 pg/ml after estradiol benzoate injection (range 34.73 to 1,016.44 pg/ml). Mean duration of estrogen surge was 20.25±1.98 h (range 12.0-26.0 h) and time of onset of estrogen surge after estradiol benzoate injection was detected at 9.62±1.03 h (range 7.0 to 16.0 h). Ovulation occurred 26.75±2.07 h (range 22.0 to 36.0 h) after end of estrogen surge in Heatsynch treated buffaloes. The profile of estrogen concentrations post estradiol benzoate injection in buffaloes, which had not ovulated (non-responders, Figure 2) was similar to that seen for buffaloes which had

ovulated to Heatsynch treatment (responders; Figure 1).

Changes in plasma progesterone profile during Heatsynch treatment : The mean plasma progesterone concentrations were basal (<0.5 ng/ml) at the time of estradiol benzoate injection in case of responding buffaloes due to luteolytic action of PGF_{2α} administration (Figure 1). However, in the non-responding buffaloes the progesterone concentrations stayed high (>2.0 ng/ml) throughout the period of sampling (Figure 2).

Timing of ovulation : Timing of ovulation (Mean±SEM) after PGF_{2α} administration, estradiol benzoate injection and end of LH surge recorded in buffaloes which responded to Heatsynch treatment are presented in Table 1. Ovulation was synchronized by estradiol benzoate injection and occurred at 50.0±2.00 h of the estradiol benzoate administration (range of 44.0 to 60.0 h; Figure 1) this corresponded to 74.0±2.0 h post PGF_{2α} administration (range of 68.0 to 84.0 h) and 18.5±2.47 h (range 8 to 26 h) post LH surge among individual buffaloes.

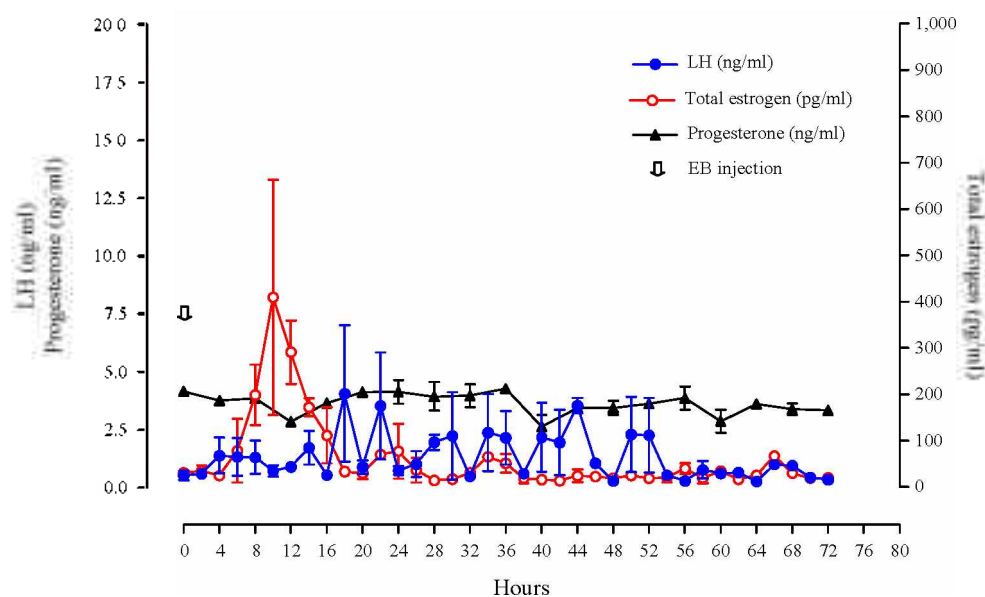


Figure 2. Changes in plasma LH, progesterone and total estrogen (Mean±SEM) profile after administration of estradiol benzoate in non-responder buffaloes (n = 2) treated with Heatsynch protocol.

Table 2. Analysis of efficacy of estrus synchronization with Heatsynch protocol and timed artificial insemination (TAI)

Treatment	Number of estruses observed	Number of estruses unobserved	Total number of estruses	Number of Animals conceived	% conceived to total estruses
Control (n = 72) (Summer)	36	36	72	13	18 ^{NS}
Heatsynch (n = 15) (Summer)	15	-	15	4	26 ^{NS}
Control (n = 101) (Winter)	78	23	101	30	30 ^{NS}
Heatsynch (n = 15) (Winter)	15	-	15	6	40 ^{NS}

Differences in the conception rates recorded among Heatsynch treated buffaloes and untreated controls were not statistically significant ($p > 0.05$).

Experiment 2 and 3

Estrus synchronization with Heatsynch protocol in terms of conception rate in summer and winter season : Comparison of conception rates of buffaloes inseminated after spontaneous estrus and estrus induced using Heatsynch protocol in summer and winter is provided in Table 2. On the basis of transrectal palpation 60 days post insemination, the conception rate was 26% in summer season whereas, conception rate was 40% in winter season for buffaloes treated with Heatsynch protocol. The percentage of conceptions to total number of estruses for control buffaloes is 18 and 30 in summer and winter respectively. The differences in the conception rates recorded among Heatsynch treated buffaloes and untreated controls were not statistically significant ($p > 0.05$).

DISCUSSION

To the best of our knowledge this is the first study conducted on estrus and ovulation synchronization in Murrah buffaloes using Heatsynch protocol. This is also the first study, which evaluated the efficacy of Heatsynch protocol for induction of estrus in terms of endocrine profile for synchronization of ovulation and timed artificial insemination in cycling Murrah buffaloes. The success rate in terms of induction of ovulation (80%) in buffaloes in the present study was higher than that reported in cows (70%) by Barros et al. (2000) using estradiol benzoate in Heatsynch protocol. Using estradiol cypionate in the Heatsynch protocol on cattle, Pancarci et al. (2002) reported similar success rate (86%) in cows, as recorded in the present study. The higher success rate in buffaloes observed in the present study using estradiol benzoate could be due to higher sensitivity of buffaloes to this treatment. However, all the treated animals responded to the treatment by displaying obvious signs of estrus. The exhibition of estrus symptoms in all animals was probably an effect of potent estradiol benzoate treatment which also resulted in all animals exhibiting high total estrogen peak concentrations in blood plasma (Figures 1 and 2). Estrus observations in all animals irrespective of success in terms of ovulation

induction clearly indicated that the occurrence of heat symptoms was a manifestation of high concentrations of estrogens and hence could not be relied upon as an indicator for ovulation occurrence in buffaloes. Earlier studies in cows (Pancarci et al., 2002; Evans et al., 2003; Cerri et al., 2004) have also demonstrated better expression using Heatsynch protocol. However, these studies have not provided information on the proportion of cows exhibiting estrus, which had ovulated.

During spontaneous estrus in Murrah buffaloes, the LH surge occurs at approximately 9.1 ± 4.4 h with a range of 0-34 h after the onset of estrus (Paul, 2003). Ovulation of a mature follicle occurs some 18-40 h later (Paul and Prakash, 2005). The use of the Heatsynch protocol results in a temporal sequence of events (estrus, LH surge and ovulation) that is essentially the same as that observed in Murrah buffaloes displaying spontaneous estrus.

Estradiol benzoate is an esterified form of estradiol 17- β that is available for use in animals in some countries. When low doses of EB are injected in cows under a low progesterone environment, it induces an LH surge from the brain (Chenault et al., 1975; Hansel and Convey, 1983; Kinder et al., 1991; Stumpf et al., 1991). A similar response has been demonstrated in cows. The duration of LH surge which succeeded the total estrogen surge in buffaloes (20.75 ± 1.99 h) seen in our study was comparable to those observed in cows (12.2 ± 0.9 h) using the Heatsynch protocol (Stevenson et al., 2004).

The timing of ovulation (50 h) in the present study in relation to estradiol benzoate injection was similar to those recorded in cows 55 h (Pancarci et al., 2002) and 59 h (Stevenson et al., 2004). Mean ovulation time in relation to LH surge of 29 h was also similar to observations recorded in cows using Heatsynch protocol; 26 h (Stevenson et al., 2004). Our study is the first to report the timing of ovulation in relation to total estrogen surge. The mean total estrogen surge in our study preceded the mean LH surge by 11 h and lasted for 20 h. The preovulatory LH surge during spontaneous ovulation is preceded by estradiol release by 12 to 24 h in cows (Stebenfeldt and Edqvist, 1996).

Similar efficacy of Heatsynch protocol in terms of

conception rates, with those observed in untreated controls in both summer and winter seasons (Table 2) suggest that the Heatsynch protocol could be very useful in buffaloes considering the fact that the incidence of silent heat in this species is very high and particularly in summers it goes up to 70% (Prakash et al., 2005; Madan and Prakash, 2007; Qureshi et al., 2008).

CONCLUSION

The results suggest the possibility of using Heatsynch treatment followed by fixed time A.I. in buffaloes for fertility improvement especially because the incidence of silent heat in buffaloes is very high particularly in summer. The Heatsynch protocol can have distinct advantage in enhancing the fertility of buffaloes especially since the technique can circumvent the need for heat detection.

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REFERENCES

- Barros, C. M., M. B. P. Moreira, R. A. Figueredo, A. B. Teixeira and L. A. Trinca. 2000. Synchronization of ovulation in beef cows (*Bos indicus*) using GnRH, PGF_{2α} and estradiol benzoate. *Theriogenology* 53:1121-1134.
- Cerri, R. L. A., J. E. P. Santos, S. O. Juchem, K. N. Galvao and R. C. Chebel. 2004. Timed artificial insemination with estradiol cypionate or insemination at estrus in high producing dairy cows. *J. Dairy Sci.* 87(11):3704-3715.
- Chenault, J. R., W. W. Thatcher, P. S. Kalra, R. M. Abrams and C. J. Wilcox. 1975. Transitory changes in plasma progestins, estradiol and luteinizing hormone approaching ovulation in the bovine. *J. Dairy Sci.* 58(5):709-717.
- Evans, A. C. O., P. O'Keeffe, M. Mihm, J. F. Roche, K. L. Macmillan and M. P. Boland. 2003. Effect of oestradiol benzoate given after prostaglandin at two stages of follicle wave development on oestrus synchronization, the LH surge and ovulation in heifers. *Anim. Reprod. Sci.* 76(1/2):13-23.
- Fernandes, P., A. B. Teixeira, A. J. Crocci and C. M. Barros. 2001. Timed Artificial insemination in beef cattle using GnRH agonist, PGF_{2α} and estradiol benzoate (EB). *Theriogenology* 55:1521-1532.
- Hansel, W. and E. M. Convey. 1983. Physiology of the estrous cycle. *J. Anim. Sci.* 57:404-423.
- Kamboj, M. and B. S. Prakash. 1993. Relationship of progesterone in plasma and whole milk of buffaloes during cyclicity and early pregnancy. *Trop. Anim. Hlth. Prod.* 25:185-192.
- Kinder, J. E., M. Garcia-Winder, K. Imakawa, M. L. Day, D. D. Zalesky, M. L. D'Occhio, T. T. Stumpf, R. J. Kittok and B. D. Schanbacher. 1991. Circulating concentrations of 17-estradiol influence pattern of LH in circulation of cows. *Domest. Anim. Endocrinol.* 8:463-469.
- Kanai, Y. and H. Shimizu. 1983. Characteristics of the estrous cycle of the Swamp buffalo under temperate conditions. *Theriogenology.* 19 (4):593-602.
- Lopes, F. L., D. R. Arnold, J. Williams, S. M. Pancarci, M. J. Thatcher, M. Drost and W. W. Thatcher. 2000. Use of estradiol cypionate for timed insemination. *J. Anim. Sci.* 78:216.
- Madan, M. L. and B. S. Prakash. 2007. Reproductive endocrinology and biotechnology applications among buffaloes. In: *Reproduction in Domestic Ruminants VI* 261-281 (Ed. J. I. Juengel, J. F. Murray and MF Smith. Nottingham University Press, Nottingham, UK.
- Mondal, M., C. Rajkhowa and B. S. Prakash. 2006. Relationship of plasma estradiol-17β, total estrogen and progesterone to estrus behaviour in mithun (*Bos frontalis*) cows. *Hormones and Behavior.* 49:626-633.
- Pancarci, S. M., E. R. Jordon, C. A. Risco, M. J. Schouten, F. L. Lopes, Moreira and W. W. Thatcher. 2002. Use of estradiol cypionate in a presynchronized timed artificial insemination program for lactating dairy cattle. *J. Dairy Sci.* 85:122-131.
- Paul, V. 2003. Studies on estrus synchronization methods, timing of ovulation and endocrine profile in buffaloes. Ph.D. Thesis, N.D.R.I., Karnal, India.
- Paul, V. and B. S. Prakash. 2005. Efficacy of the Ovsynch protocol for synchronization of ovulation and fixed-time artificial insemination in Murrah buffaloes (*Bubalus bubalis*). *Theriogenol.* 64:049-1060.
- Prakash, B. S., V. Paul and N. Anandlaxmi. 2002. Development and validation of a simple, sensitive, second antibody format enzyme immunoassay for LH determination in plasma. *J. Immunol. Methods.* 270:281-290.
- Prakash, B. S., M. Sarkar, V. Paul, D. P. Mishra, A. Mishra and H. H. D. Meyer. 2005. Postpartum endocrinology and prospects for fertility improvement in the reverine buffalo (*Bubalus bubalis*) and Yak (*Bos grunniens*). *Livest. Prod. Sci.* 98:13-23.
- Pursley, J. R., M. O. Mee and M. C. Wiltbank. 1995. Synchronization of ovulation in dairy cows using PGF_{2α} and GnRH. *Theriogenol.* 44:915-923.
- Qureshi, M. S. and N. Ahmed. 2008. Interaction among calf suckling use of oxytocin, milk production and reproduction in dairy buffaloes. *Anim. Reprod. Sci.* 106:380-392.
- Roberts, S. J. 1971. *Veterinary obstet. Gyne. and Genital Diseases* (2nd Edn.), Ithaca, New York.
- Roy, K. S. 2005. Studies on efficacy of Ovsynch protocol for estrus synchronization with or without antiprolactin (Norprolac) treatment in buffalo heifers. Ph.D Thesis, N.D.R.I., Karnal, India.
- Singh, J., A. S. Nanda and G. P. Adams. 2000. The reproductive pattern and efficiency of female buffaloes. *Anim. Reprod. Sci.* 60-61:593-604.
- Stevenson, J. S., S. M. Tiffany and M. C. Lucy. 2004. Use of Estradiol cypionate as a substitute for GnRH in protocols for

- synchronizing ovulation in dairy cattle. *J. Dairy Sci.* 87:3298-3305.
- Stumpf, T. T., M. W. Wolfe, M. L. Day, J. A. Stotts, P. L. Wolfe, J. Kittok and J. E. Kinder. 1991. Effect of 17-beta-estradiol on the preovulatory surge of LH in the bovine female. *Theriogenol.* 36:201-207.
- Sarkar, M. and B. S. Prakash. 2005. Timing of ovulation in relation to onset of estrus and LH peak in Yak (*Poephagus grunniens* L.) *Anim. Reprod. Sci.* 86(3-4):353-362.
- Stabenfeldt, G. H. and L. E. Edquist. 1996. Dukes physiology of domestic animals. In: Female reproductive processes. pp. 678-710. 11th Edition, Panima Publishing company, N. Delhi.