Hypophyseal and Gonadal Response to GnRH in Buffalo Heifers (*Bubalus bubalis*)

C. Singh[1] and M. L. Madan[2]
Embryo Technology Centre, National Dairy Research Institute, Karnal-132001, Haryana, India

ABSTRACT : The objective of this study was to investigate the responsiveness of hypophysis and gonads to synthetic GnRH among noncycling Murrah buffalo heifers at 24 months of age. The plasma FSH, LH, estradiol and progesterone levels were measured in blood samples collected at 1 hour before and upto 18th day subsequent to the administration of GnRH (200 μg) or saline (2 ml). The pretreatment levels of plasma FSH, LH estradiol and progesterone among GnRH treated heifers (N = 6) were 11.55 ± 0.57 ng/ml, 0.68 ± 0.06 ng/ml, 19.84 ± 0.82 pg/ml and 0.45 ± 0.07 ng/ml respectively. A quick elevation of FSH ($p < 0.01$) and LH ($p < 0.05$) within 5 min of GnRH administration was observed in all the heifers. The peak FSH (74.97 ± 18.63 ng/ml) and LH (3.09 ± 0.54 ng/ml) level was obtained at 30 min of GnRH administration. The elevated level of plasma estradiol on 5th to 18th day, FSH on 7th to 9th day (n = 3) and the progesterone on 13th to 18th day (n = 2) of GnRH injection was obtained. The study indicates that gonads of buffalo heifers at 24 months of age are responsive of GnRH induced gonadotropin release for folliculogenesis and luteal tissue formation
(**Key Words:** GnRH, Gonadotropin, Estradiol, Progesterone, Buffalo Heifers)

INTRODUCTION

Buffaloes being predominant dairy animals have low facundity due to long prepubertal period of 2.5 to 3.5 years (Madan et al., 1983). Hypothalamus plays the principal role through it's releasing hormone for resumption of pituitary function, the pattern of GnRH as well as gonadotropin release determine the extent of maturation of hypothalmo-hypophyseal system (Knobil 1980). The information on pattern of release of hypophyseal and gonadal hormones towards onset of puberty in buffaloes are not enough to understand the hypothlamo-hypophyseal and gonadal relationship at this age. To obtain this basic information an investigation on spontaneous release of hypophyseal and gonadal hormones as well as pattern of release of these hormones in response to GnRH administration in buffalo heifers is therefore very much emplied.

MATERIALS AND METHODS

Animals and treatments

Noncycling Murrah buffalo heifers (n = 12) at age group of around 24 months were used in this study. The animals were selected from institute's herd and were maintained under general herd managemental condition. Two hundred μg synthetic GnRH (Fertagyl) decapeptide was administrered intravenoulsy through the cactheterized jugular vein of six Murrah buffalo heifers (Experimental group) between 7 am and 9 am. Another six Murrah buffalo heifers were received sterile saline (2 ml, intravenous) at an identical time schedule. Blood samples were collected in chilled heparinized tubes from the jugular vein at 1 hour before treatment, immediately before the treatment and at 5, 10, 20, 30, 40, 60, 90 min, 2, 4, 6 and 8 hrs subsequent to GnRH and saline injections. Blood samples were also collected on 1st, 3rd, 5th, 7th, 9th, 13th and 18th day after GnRH/saline injections. The plasma was separated and divided in different aliquots and stored at −20℃ for subsequent analysis of FSH, LH, estradiol and progesterone. Genitalia were examined per rectum to observe the ovarian response and a potent vasectomised bull was used to detect the estrus.

Radio immuno assay (RIA) of hormones follicle stimulating hormone (FSH) and iuteinizing hormone (LH)

Highly purified bovine FSH (B FSH subunit, USDA-

[1] Address reprint requests to C. Singh. Vety. Physiology, B. V. C., Patna-800014, India.
[2] D. D. G. (Anim. Sci.) I. C. A. R., New Delhi-110001, India.
Received October 23, 1997; Accepted February 16, 1998

bFSH-BP3) and bovine LH (USDA bLH-I-1) was used for iodination (Niswender et al., 1969) and reference standard for FSH and LH. Double antibody RIA standardized in our lab (Palta and Madan 1995) was used for estimation of FSH and LH levels in the plasma sample.

Radio iodination of FSH and LH

Highly purified Bovine FSH (USDA-bFSH-BP3) and bovine LH (USDA bLH-I-1) was labelled with carrier free iodine Na 125 I at room temperature. Five μg bFSH and 5 μg bLH in distilled water (1 μg/ 2μl) was mixed with 10 μl 0.5 M sodium phosphate buffer (pH 7.4) in separate vial in which iodination was carried out subsequently. To this vial, 500 μCi (5 μl) of carrier free iodine ^{125}I was added and contents were mixed gently. Chloramine T, 5 μg (1 μg/2μl) in 0.05 M phosphate buffer pH 7.5 was added to the reaction mixture which was then shaken gently for 30 sec. The reaction was terminated by the addition 10 μg sodium metabisulphite (1 μg/2 μl) in 0.05 M phosphate buffer (pH 7.5). Transfer solution (100 μl) containing 16% sucrose, 10 mg/ml potassium iodide and 1 mg/10 ml bromophenol blue was added to the reaction vial.

Radio-chromatography of labelled preparation of FSH and LH

The separation of iodinated FSH and LH from free iodine was carried out on two separate Sephadex G25-80 colum (1 × 10 cm). The whole content of the reaction vial was layered on the Sephadex G25-80 column. The vial was immediately rinsed with 100 μl of rinse solution containing 8% sucrose, 10 mg/ml potassium iodide and 1 mg/10 ml bromophenol blue and the latter again layered on the column. 1.0 ml fractions were collected in tubes containing 1% BSA-PBS (0.01 M) pH 7.4. All the iodinated fractions were counted in Auto Gamma Counter.

Typical elution pattern on Sephadex G25-80 showing the separation of bFSH ^{125}I and bLH ^{125}I from free ^{125}I has been shown in figure 1. The first peak was of ^{125}I FSH and ^{125}I LH whereas the second peak represented free ^{125}I. The fraction of tube No 5 showing the first peak of both FSH and LH divided in aliquots and stored at −20℃.

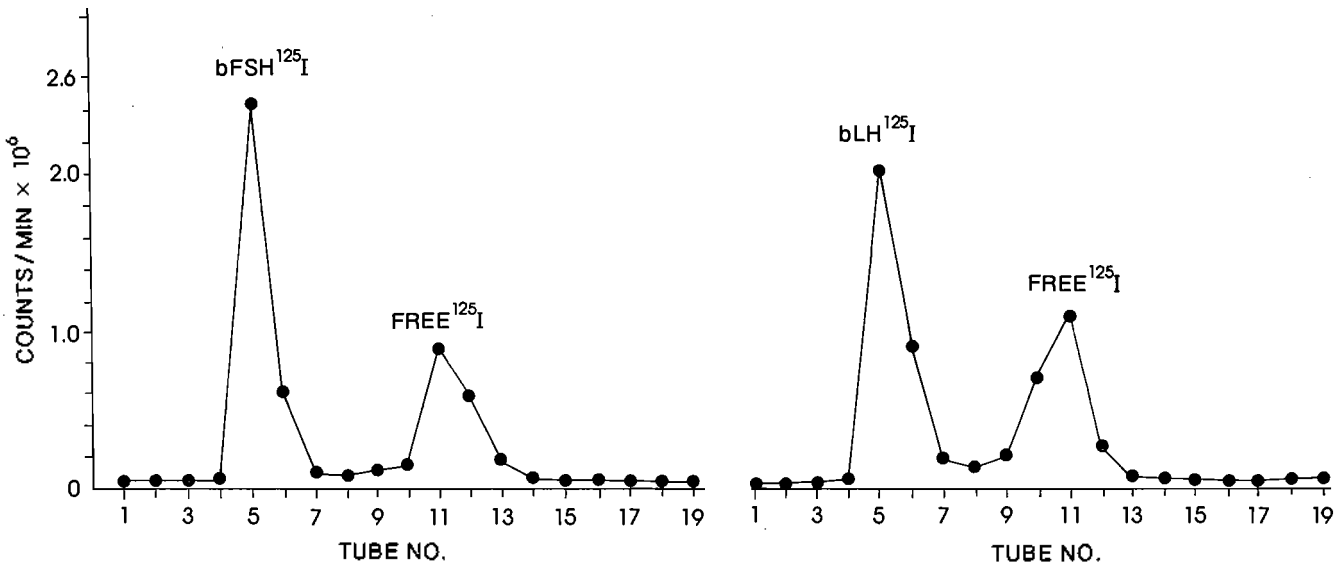

Figure 1. Elution diagram of reaction mixture for bovine follicle stimulating hormone and luteinizing hormone iodiation (^{125}I).

Assay protocol of FSH

Plasma samples (0.1 ml) were pipetted in disposable glass tubes (10 × 75 mm). Simultaneously, a series of standards ranging from 0.04 to 40 ng were also pipetted. 0.4 ml of 1% BSA-PBS (pH 7.0) was added to each tube. The FSH antiserum was diluted to 1 : 15,000 with 0.05 M EDTA-PBS (pH 7.0) and 0.2 ml diluted antiserum was added to all the tubes. The tubes were than vortexed and incubated at refrigerator temperature (4℃) for 24 hr. 0.1 ml of labelled ^{125}I FSH (10,000 CPM) was then added to all the tubes. The tubes were vortexed and incubated for another 24 hr at 4℃. Following this incubation, appropriately diluted 0.2 ml of goat anti-rabbit gamma globulin (in NRS-EDTA-PBS) was added to all the tubes.

The contents of the tubes were vortexed and again incubated for 48 hr in refrigerator at 4℃. Following final incubation, cold PBS (1.0 ml) was added to dilute unbound hormone radioactivity. The antibody bound hormone complex was separted from free labelled hormone by centrifuging at 3,000 rpm for 45 min at 4℃. The supernatant was decanted and assay tubes were kept inverted on the absorbent paper. The assay tubes were subsequently counted in Auto Gamma Counter Programmed for hormone quantitation.

Assay protocol of LH

Duplicate plasma samples (0.1 ml) were pipetted in glass test tubes (10 × 75 mm). Simultaneously, a series of standard ranging from 0.01 to 10 ng were also pipetted. 0.4 ml of 1% BSA-PBS (0.01 M, pH 7.0) was added to each tube. 0.2 ml of bLH antiserum (1 : 1,000,000) diluted with 0.05 M EDTA-PBS (pH 7.0) was added to all the tubes. The tubes were then vortexed and incubated at 4℃ for 24 hr. Thereafter, 0.1 ml tracer ^{125}I-LH (10,000 CPM) was added to each tube following which the tubes were vortexed and incubated for another 24 hrs at 4℃. Following this incubation, appropriately diluted 0.2 ml of goat anti-rabbit gamma globulines in NRS-EDTA-PBS (Non-immune rabbit serum was diluted to 1:300 with 0.05 M EDTA-PBS, pH 7.0) was added to all the tubes, vortexed and incubated again for 48 hr at 4℃. Cold PBS (2.0 ml) was added following the final incubation to dilute unbound hormone radioactivity. The antibody bound hormone complex was separated from free hormone by centrifuging at 3,000 rpm for 45 min at 4℃. The supernatant was decanted and assay tubes were kept inverted on the absorbent paper to drain of left over supernatant. The assay tubes were subsequently counted in Auto Gamma counter programmed for hormone quantitation.

Assay protocol of estradiol

RIA based on the method of Echterncamp et al. (1976) was used for estimation of estradiol in plasma. Estradiol purchased from Sigma chemical company, St-Louis, USA and antiserum (Estradiol DJB 186-2) was gifted by Dr. D. J. Bolt, Animal Science institute, Beltsvile, Marryland, USA and labelled estradiol (2, 4, 6, 7-^{3}H Estradiol 17^{B}) received from Amersham International PLC Amersham, U. K. were used for estradiol estimation. The estradiol was extracted from 1,000 μl plasma for one minute with 5 ml diethlyl ether twice. The ether was evaporated to dryness. The residue was dissolved in 500 μl 0.1 M sodium phosphate buffer (pH 7.0) and 100 μl labelled estradiol (10,000 CPM) was added, vortexed and incubated at 4℃ for 10 hours. 100 μl of antiserum (1 : 36,000) was then added to all tubes. The tubes were vortexed and incubated in water bath at 40℃ for 30 minutes. The tubes were then transferred to ice water and incubated for 90minutes. Five hundred μl of cold charcoal mixture (0.625% activated charcoal and 0.0625% dextran suspension) under constant stirring was added to each tube. Tubes were then centrifuged at 3,000 rpm at 4℃ for 10 minutes. The supernatant containing the antisera bound estradiol was decanted into glass scintillation vials. Ten ml of scintillation fluid (0.1 g popop and 4.0 g ppo in 1,000 ml toluene) was poured in all the vials and vials were tightly capped. The mixture was left for 12 hours at room temperature. The hormone concentration in each vial was determined by liquid scintillation counter (LKB) programmed for microquantitation of hormones.

Assay protocol of progesterone

RIA based on the method of Prakash and Madan (1986) was used for estimation of progesterone in blood plasma. Progesterone purchased from Sigma chemical Company, st Louis USA and the progesterone monoclonal antibody (P_5 D_6 F_2 reacted preferably with progesterone) used was gifted by Dr. G. P. Talwar, Director, National Institute of Immunology, New Delhi.

Duplicate 0.2 ml plasma were taken into 12 × 100 mm glass tubes and extracted with 2 ml diethyl ether for 1 min twice. The lower aquous layer was frozen in the ice salt mixture and the upper organic layer was decanted in 12 × 100 mm glass test tubes. The ether was evaporated to dryness in a water bath. The walls of the tubes were washed with 1 ml ether and dried again. The residue was dissolved in 0.5 ml of 0.05 M phosphate buffer saline pH 7.4, vortexed for 1.0 min and incubated in water bath for 30 min at 40℃ for complete solubilization of steroids. Subsequently 0.1 ml of suitably diluted monoclonal antibody (1 : 15,000) was added to each tube followed by the addition of 0.1 ml of tracer (10,000 CPM). The resulting mixture was vortexed for 30 sec and incubated at 4℃ in refrigerator for 20 hrs. Following incubation, 0.2 ml of freshly prepared mixture of 0.625% activated charcoal and 0.0625% dextran under constant stirring in cold condition was rapidly added to each tube. The tubes were vortexed and kept for 5 min in ice water and thereafter centrifuged at 3,000 rpm for 10 min at 4℃. The supernatant containing the bound progesterone was decanted into scintillation vials. 10 ml of scintillation fluid (0.1 g popop, 4.0 g ppo in 1,000 ml toluene) was poured in each scintillation vial and were kept overnight tightly capped at room temperature. The radio activity and hormone concentration were determined in each vial

through programmed liquid scintillation counter (LKB).

The sensitivity of FSH, LH, estradiol and progesterone assay was 0.08 ng/tube, 0.02 ng/tube, 2.50 pg/tube and 20 pg/tube respectively. The intra-and inter-assay coefficient of variation for FSH were 4.5% and 4.9%, for LH were 5.1% and 6.1% for estradiol were 9.2% and 8.8% and for progesterone were 4.9 and 5.7% respetively.

The pair 't' test to compare the difference of mean of FSH, LH, estradiol and progesterone pre and post GnRH treatment and two way analysis of varience to compare the difference of means of hormones were obtained according to the methods described by Snedecor and Cocharan (1967).

RESULTS

The pretreatment basal plasma FSH (11.55 ± 0.57 ng/ml) and LH (0.68 ± 0.06 ng/ml) concentration of experimental group was similar to the FSH (12.18 ± 0.61 ng/ml) and LH (0.81 ± 0.06 ng/ml) concentration of control group heifers. A quick rise (Mean ± SE) of Plasma FSH (33.87 ± 7.72 ng/ml) and LH (1.75 ± 0.31 ng/ml) concentrations within 5 min after GnRH administration was detected in experimental group. The peak (Mean ± SE) level of plasma FSH (74.97 ± 18.63 ng/ml) and LH (3.09 ± 0.54 ng/ml) reached by 30 min after GnRH injection. Thereafter the concentration of FSH and LH declined gradually and returned to basal value by 4 hours and 6 hours after GnRH injection respectively (figure 2).

The pretreatment plasma estradiol (19.84 ± 0.82 pg/ml) and progesterone (0.45 ± 0.07 ng/ml) concentration of experimental group was similar to the plasma estradiol (21.61 ± 0.69 pg/ml) and progesterone (0.42 ± 0.06 ng/ml) concentration of control group heifers. The estradiol concentration increased significantly ($p < 0.05$) on 5th to 7th day which was followed with coincident increase of plasma FSH levels ($p < 0.05$) on 5th (16.08 ± 2.63 ng/ml), 7th (20.00 ± 4.67 ng/ml) and 9th (16.48 ± 3.57 ng/ml) day after GnRH administration. The estradiol concentration declined on subsequent days but remained elevated above pretreatment value even on 18th day after GnRH administration (table 1). The progesterone concentration also increased over pretreatment value on 9th to 18th day (table 1). The increase in mean plasma concentration of estradiol on 5th to 7th day, FSH on 5th to 9th day and progesterone on 9th to 18th day after GnRH administration was due to increased release of these hormones in three heifers during respective period

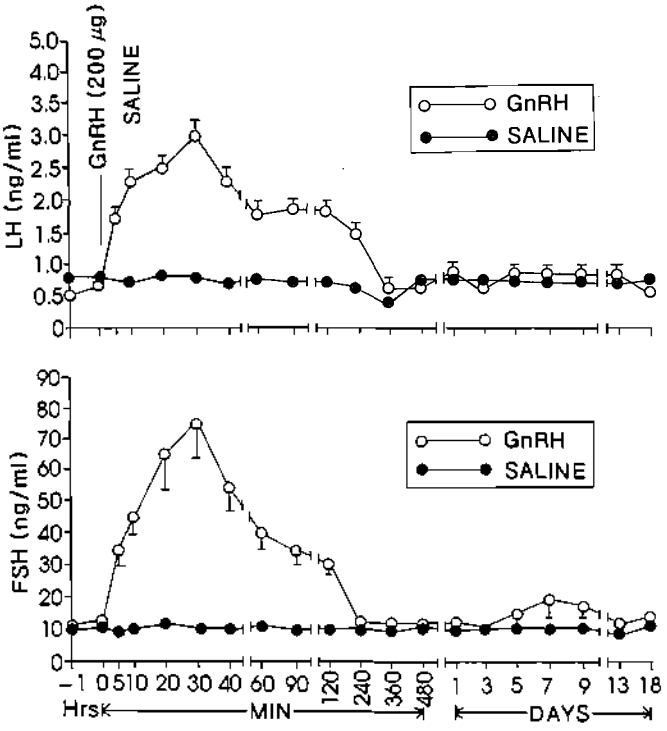

Figure 2. Plasma (Mean ± SE) FSH and LH release following GnRH administration in 24 months old buffalo heifers.

Table 1. Plasma (Mean ± SE) estradiol and progesterone concentraion following GnRH administration (200 µg, 0 day) in 24 months old buffalo heifers

Time	Estradiol(Pg/ml)		Progesterone (ng/ml)	
	Treatment (GnRH)	Control (Saline)	Treatment (GnRH)	Control (Saline)
0 day	19.84 ± 0.82	21.61 ± 0.68	0.45 ±0.07	0.42 ±0.06
1st day	21.31 ± 0.92	21.05 ± 1.28	0.47 ±0.06	0.41 ±0.05
3rd day	23.40 ± 1.52	22.34 ± 0.66	0.53 ±0.06	0.43 ±0.04
5th day	31.00 ± 4.19	21.09 ± 1.16	0.55 ±0.07	0.39 ±0.03
7th day	46.84 ±14.42	20.69 ± 1.04	0.47 ±0.06	0.39 ±0.02
9th day	25.21 ± 3.01	21.75 ± 0.34	0.62 ±0.08	0.41 ±0.02
13th day	25.93 ± 3.93	21.81 ± 0.83	0.87 ±0.18	0.40 ±0.02
18th day	29.96 ± 9.77	21.14 ± 0.83	0.82 ±0.25	0.37 ±0.04

after GnRH administration. The administration of saline did not cause alteration of plasma FSH, LH, estradiol and progesterone level among control group animals.

DISCUSSION

The basal FSH level detected in 24 months old buffalo heifers in present study was higher than basal FSH value recorded in 12 months buffalo heifers (Singh and Medan 1997) but comparable to the basal FSH value recorded in anestrus Murrah buffalo cows (Palta and Madan 1995). This suggests that coordination of hypothalamic and hypophyseal activities are resumed in 24 months old buffalo heifers in a fashion as established in mature cycling animals (Hafez 1987). The GnRH response obtained in this study in respect to amplitude and duration of FSH release was similar to the response recorded in GnRH treated heifers (Mc Leod et al., 1984), prepubertal buffaloes (Singh and Madan 1997) and at different post partum interval of Murrah buffaloes (Palta and Madan 1995). A subsequent rise of estradiol (5th day onward), FSH (5th to 9th day) and progesterone (9th to 18 day) might be due to the intrinsic rhythm initiated by elevated levels of FSH and LH in response to GnRH administration in three heifers. The GnRH induced release of FSH and LH might have triggered the mechanism of follicular development and estradiol secretion on 5th day onwards in these heifers. This early rise of plasma estradiol concentration and coincident elevation of FSH reveals that the feed back mechanism is also established in buffaloes at 24 months of age identical to the mature cycling animals (Hafez 1987).

The pretreatment plasma LH values obtained in 24 months old buffalo heifers was identical to the values recorded in buffalo heifers of similar age (Jain and Pandey 1985) but lower than the values obtained in mature buffaloes (Avenell et al., 1985). However, the pattern of LH release in response to GnRH in present experiment was similar to the trends reported in prepubertal heifer (McLeod et al., 1984).

The circulating levels of plasma progesterone recorded in 24 months old buffalo heifers was comparable to the values recorded in Murrah buffalo cow (Madan et al., 1983) while higher than the values reported in 12 months old Murrah buffalo heifer (Singh and Madan 1997) indicating that the buffalo heifers at 24 months of age have achieved sexual maturity as increase of plasma progesterone concentration towards the onset of puberty and sexual maturity in heifers has been well documented (Gonzalez-Padilla et al., 1975). The plasma estradiol concentration recorded in present experiment was comparable to the plasma estradiol concentration recorded in heifers (Gonzalez-Padilla et al., 1975a) and buffalo heifer (Jain and Pandey 1985, Singh and Madan 1997).

Two heifers exhibiting higher estradiol on 5th and 7th day of GnRH treatment were detected in estrus at 180 hours of GnRH administration. Moreover, duration of estrus was very short (5 to 6 hours). The less intense and shorter duration of estrus is common in buffaloes (Dobson and Kamonpotana 1986). Similar to present observation single injection of GnRH in buffaloes (Ray et al., 1995) caused follicular growth and ovulation leading to corpus luteum formation.

Thus it can be concluded that hypothalmo-hypophyseal and gonadal axis is mature in buffalo heifers at 24 months of age for feedback sensitivity similar to the mature animals. The gonadotrophs have ready releasable pools of FSH and LH and await for the hypothalamic hormone for its release. The gonads at this age are responsive to tropic hormones for folliculogenesis, ovulation and corpus luteum formation.

ACKNOWLEDGEMENTS

We are grateful to Dr. D. J. Bolt, Animal Sci. Inst. USDA Beltsville, MD, USA for supply us purified preparations of LH, FSH and estradiol and to Dr. G. P. Talwar, Director, NII, New Delhi, India for generous gift of progesterone monoclonal antibody.

REFERENCES

Avenell, J. A., Y. Sacpudin and I. C. Fletcher. 1985. Concentration of LH, oestradiol-17B and progesterone in the pripheral plasma of Swamp buffalo. J. Reprod Fert. 74:419-424.

Dobson, H. and M. A. Kamonpotana. 1986. Review of female cattle reproduction with special reference to comparison between buffaloes, cows and zebu. J. Reprod Fert. 77:1-36.

Echternkamp, S. E., D. F. Bolt and H. W. Hawk. 1976. Ovarian and pituitary hormones of blood of progestogen-treated ewes. J. Animal Sci. 42:893-900.

Gonzalez-padilla, E., G. D. Niswender and J. N. Wiltbank. 1975. puberty in beef heifers I. Effect of injections of progesterone and estradiol-17B on serum LH, FSH and overian activity. J. Anim. Sci. 40:1105-1109.

Gonzalez-padilla, E., J. N. Wiltbank and G. D. Niswender. 1975a. Puberty in beef heifers. The interrelationship between pituitary, hypothalamic and ovarian hormones. J. Anim. Sci. 40:1091-1104.

Hafez, E. S. E. 1987. Reproduction in farm animals. 5th Edn Lea and Fibigar, pheladalphia.

Jain, G. C. and R. S. Pandey. 1985. Influence of age, weaning, season, body weight on the levels of progesterone, estradiol-17B luteinizing hormone in growing buffalo heifers (*Bubalus bubalis*) Anim Reprod. Sci. 9:213-221.

Knobil, E. 1980. The neuroendocrine control of the menstrual cycle. Recent prog Horm Res. 36. 53-58.

Madan, M. L., S. M. K. Naqvi, C. V. Triu, A. K. Suri and B. S. Paraksh. 1983. Plasma estradiol-17B, progesterone and cortisol among anestrus rural animals. In symposium on Animal Reproduction, society for study of Animal Reproduction, HAU, Hissar, India, February.

McLeod, B. J., W. Haresign, A. R. Peters and G. E. Lamming. 1984. Plasma LH and FSH concentrations in prepubertal beef heifers before and in response to repeated injection low doses of GnRH. J. reprod Fart. 70:137-144.

Niswerder, G. D., L. E. Reichert Jr., A. R. Midgley Jr. and A. V. Nalvandav. 1969. Radioimmuno assay for bovine luteinizing hormone. Endocrinology 84:1166-1173.

Palta, P. and M. L. Madan. 1995. Alterations in hypophysial responsiveness to synthetic GnRH at different postpartum intervals in Murrah buffalo (*Bubalus bubalis*). Theriogenology. 44:405-411.

Prakash, B. S. and M. L. Madan. 1986. Peripheral plasma oestradiol-17B, progesterone and cortisol in buffaloes induced to calve with dexamethasone and vetoesterol. Anim. Reprod. Sci. 11:111-122.

Ray, G. P., M. H. Akhtar, A. P. Singh, K. M. Prasad and R. B. Singh. 1995. Effect of "Receptal" to improve the fertility in bovines. Ind. J. Anim. Sci. 16:13 Abstr.

Singh, C. and M. L. Madan. 1997. Pituitary and gonadal response to GnRH in prepubertal buffaloes (*Bubalus bubalis*). Asian Australasian J. Anim. Sci. 11:78-83.