Characterization of Seasonal Variations in Responsiveness of Pituitary Gland to Different Doses of Gonadotropin Releasing Hormone in Buffalo Cows

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Abstract: In tropical countries such as India, it has been observed that a number of buffalo cows experience seasonal anestrous during summer months. This might be due to seasonal changes in responsiveness of pituitary gland to gonadotropin releasing hormone (GnRH) and/or decreased hypothalamic GnRH release. Attempts were made to characterize the responsiveness of pituitary gland to a range of doses (0.1, 1, 3, 10 and 33 µg) of GnRH in terms of LH and progesterone (P_4) secretions during summer (April-May) and rainy (September-November) months. As a part of these studies, a radioimmunoassay method for estimation of circulating LH in buffalo cows was standardized. During summer months, it was observed that in the presence of low circulating P_4 levels the minimum dose of GnRH required for eliciting a significant increase in circulating LH levels was 10 µg/animal that corresponded to a dose of ~28 ng/kg BW. However, during rainy months, administration of the same dose of GnRH failed to elicit a response suggesting that the pituitary gland is not responsive to low doses of exogenous GnRH. On the other hand, buffalo cows receiving a dose of 100 µg of GnRH during rainy months elicited a surge-like increase in circulating LH that peaked at 2 h and the increase in LH concentrations lasted for nearly 6 h post GnRH treatment. The results appear to suggest that during summer months the pituitary gland function is not affected, but there may be lowered hypothalamic GnRH input to the pituitary gland.

Keywords: GnRH, LH, FSH, buffalo cows, Holstein-Friesian, rainy season, summer season, hypothalamus, estrous cyclicity, artificial insemination.

INTRODUCTION

Of the neuroendocrine peptides that play critical role in the control of reproduction in vertebrates, the decapeptide gonadotropin releasing hormone (GnRH) also termed luteinizing hormone releasing hormone (LHRH) for its preferential action on LH secretion, is the key molecule that acts on pituitary gonadotropes to stimulate the secretion of gonadotropins *viz*., LH and follicle stimulating hormone (FSH), and these hormones in turn act on the ovary to stimulate its steroidogenesis and gametogenesis. Generally, the ovarian steroids act on the hypothalamus to regulate GnRH secretion. Also, the ovarian steroids and inhibin act directly on the pituitary gland to modulate the sensitivity of gonadotropes to GnRH. It is now well established that pulsatile secretion of GnRH is essential for the pulsatile release of LH from pituitary gland into circulation. The pulsatile LH secretion is used as an indirect measure of hypothalamic GnRH secretion [1]. The pulsatile secretion of LH, and therefore GnRH secretion, has been shown to fluctuate throughout the reproductive cycle depending on steroid status ranging from one pulse per hour during the follicular phase to one pulse every 3-4h or longer during the luteal phase [2, 3].

The dose and frequency of exogenous GnRH administration have marked effects on the pituitary gonadotrophin secretion. Continuous administration of GnRH leads to desensitization of the pituitary gland responsiveness to GnRH leading to suppression of LH secretion, down regulation of GnRH receptor (R) and decrease in mRNA expressions of GnRHR and LHβ subunit [4, 5, 6]. Cows experiencing anestrus induced by nutrition deficit or those cows that experience postpartum anestrus reported to have reduced number and amplitude of LH pulses with low circulating LH levels [7 - 9]. Thus, different animal models have been employed to evaluate the dose, frequency and duration of exogenous GnRH required to stimulate pulse secretion of LH [10 - 13]. Studies in anestrus suckled cows indicated that administration of 0.5 µg of exogenous GnRH once every h or 2 h or GnRH agonist (15 µg) administered as microencapsulated form resulted in increased LH pulse frequency and decreased amplitude [11, 12]. Studies employing nutrition deficit induced anestrus beef cows have shown that administration of GnRH at hourly intervals resulted in higher LH pulse frequency and the amplitude of LH pulse increased with increasing doses of GnRH employed. These studies suggest that

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decreased LH secretion in nutritionally anestrus animals may be due to reduced GnRH release from the hypothalamus [6, 13].

Seasonal variations in environment, nutrition, and management alter incidence of estrous cyclicity in cattle and buffalo cows. It has been documented that increased number of animals exhibited regular estrous cycles and increased conception rate during rainy and winter seasons compared to spring and summer seasons [14, 15]. Studies on seasonal variations in cyclicity have focussed on the effect of heat stress, temperature and humidity on ovarian function and use of various synchronization protocols to improve conception rates during summer months [16-19]. In cattle, heat stress during summer causes a decrease in amplitude of LH pulses and the GnRH-induced surge concentrations of LH and FSH are lower during summer than in winter [20]. A reduced LH pulse frequency has been observed during the luteal phase as well as reduction in both LH pulse frequency and amplitude during the follicular phase in summer compared to winter in buffalo cows [21]. Also, the lower LH peak values and circulating FSH levels were observed in summer months [22, 23]. To date, the responsiveness of the pituitary gland to exogenous GnRH administration during summer and winter months and the dose of exogenous GnRH required for eliciting a surge-like increase in circulating LH levels in buffalo cows remains to be established. Thus, the objectives of the present study were to: (i) standardize an assay system to quantitate circulating LH levels in buffalo cows (ii) determine the dose of exogenous GnRH required to elicit a physiological pulse-like increase in circulating LH levels (iii) characterize and compare the response of the pituitary gland to a range of doses of exogenous GnRH during summer and rainy months, and (iv) to determine the dose of exogenous GnRH required to elicit a surge-like increase in circulating LH levels leading to ovulation during rainy months (breeding season) in buffalo cows.

MATERIALS AND METHODS

Reagents

GnRH (LHRH) was procured from Sigma-Aldrich, Bangalore, India. Anti-bovine LH antiserum (Lot# USDA-309-684P) and iodination grade bovine LH (AFP11743B) were procured from Dr. A.F. Parlow, National Hormone and Peptide Program (NHPP), Harbor-UCLA Research and Education Institute, CA, USA. All other reagents were purchased from Sigma-Aldrich, Bangalore, India.

Analysis of Reproductive Cyclicity Data Throughout the Year in Buffalo Cow

With a view to determine whether fewer animals exhibited estrous cycles during summer months, we analysed information of reproductive cyclicity data such as number of buffalo cows reporting every month for artificial insemination service from one of the veterinary service centres in the state of Karnataka, India. The cyclicity data available for analysis were for years 2003-2005. For comparison purpose, we also analysed monthly cyclicity data information for Holstein-Friesian cross bred dairy cows for years 2003 and 2004. The data is presented as total number of buffalo cows/cross bred cows reporting for AI service. From the cyclicity data, whether the same animal was reported for AI more than once was not determined.

Effect of Different Doses of GnRH Treatment on LH Secretion During Summer and Rainy Months

Six non lactating buffalo cows were used for the studies.The experimental protocol for GnRH challenge experiment involved intravenous administration of different doses of GnRH *viz*., 0.1, 1, 3, 10 and 33 µg to buffalo cows. Blood samples were collected immediately before, 30, 60, 120, 180 and 240 min after intravenous injection of GnRH. Based on the body weights, the doses of GnRH corresponded to ~ 0.28, 2.8, 8.4, 28 and 92.4 ng/kg BW for 0.1, 1, 3, 10 and 33 µg of GnRH employed. The same sets of animals were used for studying the response to different doses of GnRH during summer and rainy months. The experiments were carried out in the year 2007. The temperature during April and May months ranged from a minimum of 18.2°C (April)/ 20°C (May) to maximum of 36.1°C (April)/ 35.6°C (May) and the temperatures were 19°C/31°C, 18°C/32°C and 13°C/29°C for September, October and November months, respectively.

Experiments During Summer Months

During summer months of April and May 2007, buffalo cows were monitored for circulating P_4 levels over a period of 3 weeks to assess the ovarian function. After confirming for presence of low P_4 levels (<1 ng/ml 3 weeks before and at the time of start of the experiment) indicative of absence of luteal function, the animals were assigned to receive different doses of GnRH in random order. Doses of GnRH administered with the number (n) of animals receiving each dose of GnRH indicated in the parenthesis are as follows: 0.1 (n=4), 1 (n=3), 3 (n=2), 10 (n=5) and 33 (n=1) µg of

GnRH. An interval of at least one week separated between each dose tested within the animal. Blood samples collected at different time intervals were assayed for P_4 and LH.

Experiments During Rainy Months

During rainy months of August, September and October 2007, buffalo cows were monitored for circulating P_4 levels over a period of 3 weeks to assess the ovarian function. After confirming for presence of high circulating P_4 levels (≥ 1 ng/ml) indicative of luteal activity, the animals were assigned to receive different doses of GnRH in random order. Doses of GnRH administered intravenously with the number of buffalo cows receiving each dose of GnRH indicated in the parenthesis, are as follows: 0.1 (n=3), 1 (n=3) and 10 (n=5) µg of GnRH. An interval of at least one week separated between the each dose tested within the animal. Blood samples were collected at different time intervals were assayed for P_4 and LH.

Effect of a Large Bolus of GnRH on LH Secretion in Buffalo Cows

In cattle, it is well established that administration of a bolus of GnRH during the preovulatory period results in surge-like release of LH and FSH [24]. In this experiment, effect of intramuscular injection of 100 µg of GnRH on LH secretion was examined in buffalo cows. After confirming for presence of high circulating P_4 levels and thus functional corpus luteum, three animals received intramuscular injection of 100 µg of GnRH, and blood samples were collected immediately before and at hourly intervals for 6 h post GnRH injection.

Hormone Assays

Serum P_4 concentrations were determined by specific radioimmunoassay as reported previously [25].

Radioimmunoassay (RIA) for LH

Serum LH concentration was determined by employing bovine LH assay reagents. The protocol for LH assay standardized in the laboratory is outlined below. The composition of RIA buffer in which hormone and antiserum were diluted contained 0.05M sodium dihydrogen phosphate (phosphate buffer), 4.5 mM EDTA and 150 mM NaCl and 0.1% of gelatin. Bovine LH was radio-iodinated according to Sairam *et al*., [26] with some modifications in the present study. To 5 µg of the hormone, 0.5 mCi ^{125}I in 20 µl distilled water and 25 µl of 0.05 M phosphate buffer were added. After gentle mixing and incubating for 50 sec, 10 µl of Chloramine-T (15 µg) solution in 0.05 M phosphate buffer was added and the reaction was terminated by adding 30 µl of sodium metabisulphite (45 µg) solution. The iodinated hormone was separated on Sephadex G-25 (coarse) column. Serial dilutions of iodination grade bovine LH antigen was prepared in 0.05 M phosphate buffer containing 0.9 % NaCl and the concentration range of bLH used to prepare the standard curve was: 0.01, 0.02, 0.04, 0.08, 0.1, 0.2, 0.4, 0.8, 1.0, 2.0 ng/tube. Anti-bovine LH antiserum diluted (1:30,000) in RIA buffer containing 1:100 normal rabbit serum was added to each tube. The assay contained tubes for measuring non-specific binding (NSB; RIA buffer + labeled LH) and maximum binding (B_0; contained RIA buffer + antiserum + labeled LH). To the NSB tubes, 100 µl of NRS-EDTA-PBS was added and for rest of the tubes 100 µl of 1:30,000 anti-bovine LH antiserum, 100 µl of standard or sample was added to the respective tubes and the total volume was made up to 400 µl in each tube by addition of 0.05 M RIA buffer. The tubes were vortexed and incubated at room temperature for 2 days. Later, 50 µl of iodinated bovine LH containing 20,000 cpm was added to each tube, vortexed and incubated for 24 h at room temperature and at the end of the incubation, 100 µl of 1:2 diluted rabbit double antibody was added, vortexed and incubated further for 24 h. The assay was terminated by adding 3 ml ice-cold 1X PBS. The tubes were centrifuged at 2600 g at 4^0C for 30 min. The supernatant was decanted and the pellet counted in gamma counter (Wallac Wizard^{TM} 1470 automatic, Perkin Elmer, Massachusetts, USA). The standard curve was constructed using cpm obtained for different hormone concentrations. The sensitivity of the assay was 0.02 ng/tube, and inter- and intra-assay coefficients of variation were <10%.

Statistical Analysis

Data was expressed as mean ± SEM. LH data analyzed by ANOVA followed by Neuman-Keuls multiple comparison test (PRISM graph pad v2, Graphpad software, Inc. USA). A 'P'value of< 0.05 was considered statistically significant.

RESULTS

Analysis of reproductive cyclicity data

Figure **1** shows number of animals reported for AI service for buffalo cows (Surthi breed) and Holstein-Friesian cross bred cows. In buffalo cows, as can be seen from the Figure **1**, the number of animals (also

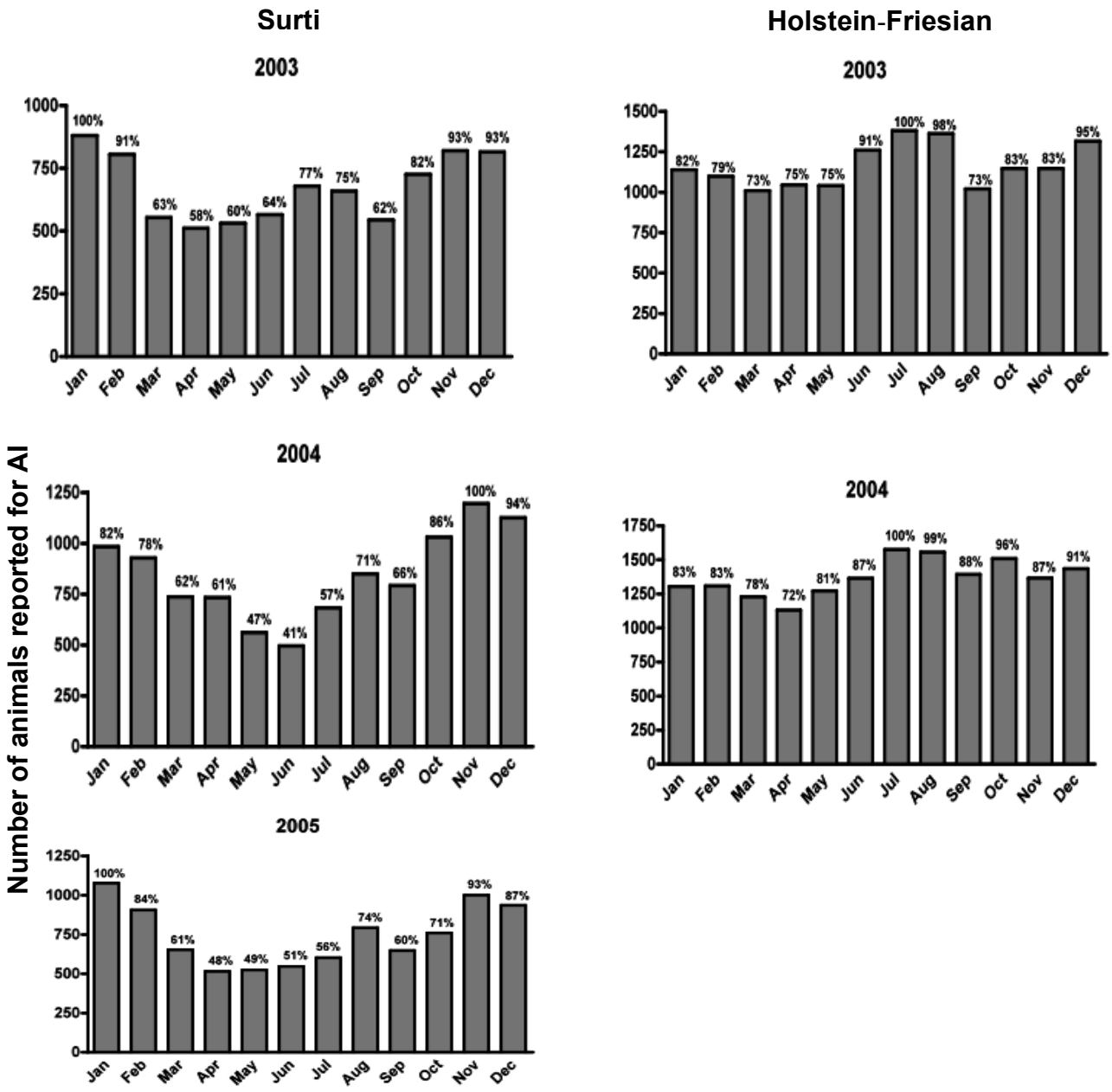

Figure 1: Bar diagram representation of number of buffaloes (Surti breed) and Holstein-Friesian cross bred cows reporting for AI service every month in the year 2003-2005 in one of the veterinary AI service centres in Karnataka, India. In each year, the maximum number of animals reporting for AI in a given month was set as 100% and the number of animals reporting for other months was expressed in relation to 100%. The calculated percent of animals reporting for AI for each month is shown on individual bar graph.

represented as % of animals) reported for AI are lower during summer months compared to rainy and winter months.

Characterization of Pituitary Response to Exogenous GnRH Treatment During Different Seasons

a) Summer Months

Circulating P_4 levels were monitored in buffalo cows for 3 weeks prior to the beginning of GnRH challenge experiment and immediately before administration of GnRH. Serum P_4 levels were 0.66 ± 0.3 ng/ml and 0.36 ± 0.1 ng/ml three weeks prior to and at the start of GnRH challenge experiment, respectively in buffalo cows. The circulating levels of LH in response to GnRH challenge experiment in buffalo cows is illustrated in Figure **2**. Administration of GnRH at doses of 0.1, 1 and 3 µg (data not shown) did not result in a significant increase in circulating LH over basal levels (Figure **2**, left panel). However, administration of 10 µg of GnRH

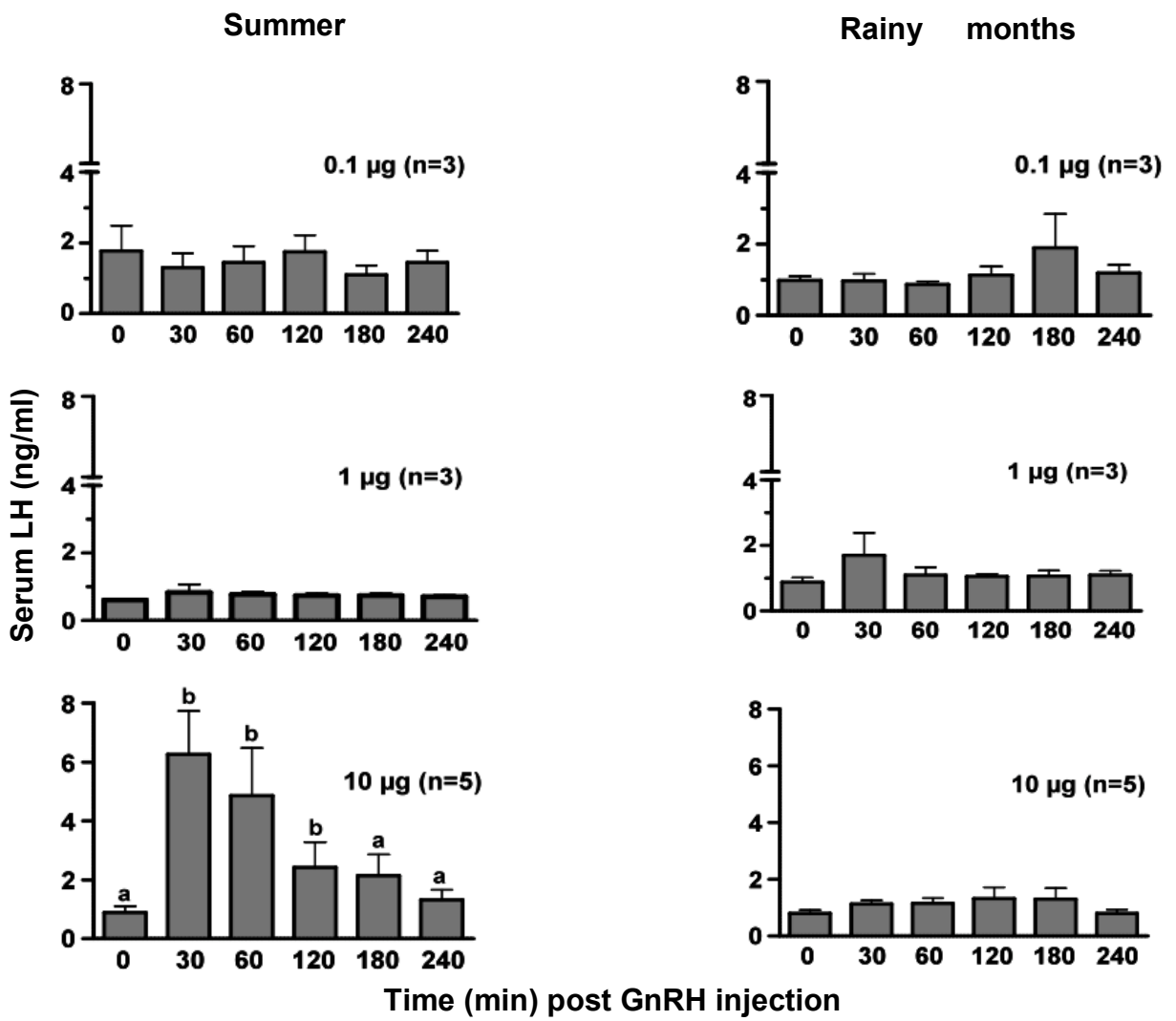

Figure 2: Mean (± SEM) of serum concentrations of LH in buffalo cows before and after administration of different doses of GnRH.

resulted in a significant increase in circulating concentration of LH from basal levels of 0.88 ± 0.23 ng/ml to 6.27 ± 1.8 ng/ml at 30 min post GnRH treatment. This was followed by a gradual decrease at later time points to reach basal levels by 240 min. Administration of 33 µg of GnRH resulted in a further increase in circulating LH levels from basal levels of 0.46 ng/ml up to 10 ng/ml by 30 min followed by a gradual decline and the circulating LH level remained above the pre-treatment level.

The dose response curve for GnRH vs fold increase in circulating LH levels demonstrated that a higher dose of GnRH was required to reach plateau in terms of increase in circulating LH levels (Figure **3**).

b) Rainy Months

Concentrations of P_4 in circulation were monitored and the P_4 levels were 1.2 ± 0.4 ng/ml and 1.8 ± 0.2 ng/ml in buffalo cows for 3 weeks prior to and during the GnRH challenge experiment. Administration of GnRH at doses of 0.1, 1 and 10 µg did not result in a significant increase in circulating LH over the basal levels (Figure **2**, right panel).

Circulatory LH Secretion in Response to Large Bolus of GnRH Treatment

Administration of 100 µg dose of GnRH resulted in increased circulating LH from basal levels of 0.83 ± 0.27 to 15.3 ± 1.6 ng/ml by 1 h, and the levels were 27.6 ± 5.3 ng/ml at 2 h post GnRH administration followed by a gradual decrease by 4 h post GnRH administration (Figure **4**).

DISCUSSION

It has been well documented that during spring and summer months, fewer number of animals return to

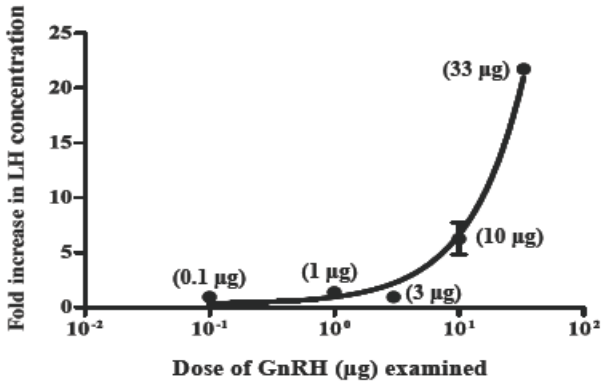

Figure 3: Effects of different doses of GnRH on LH secretion in buffalo cows during summer.

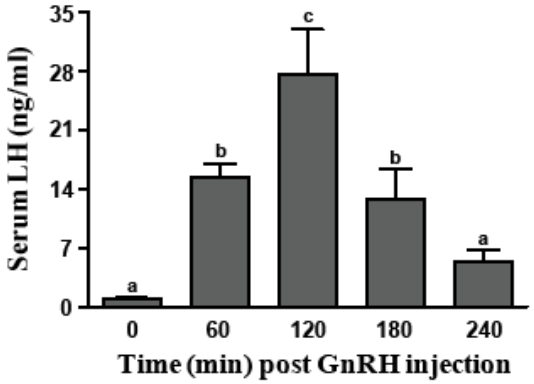

Figure 4: Mean (± SEM) of serum concentrations of LH in buffalo cows before and after administration of 100 µg GnRH.

estrus compared to winter and rainy seasons in cattle and buffalo cows [14, 27, 28]. The seasonal variations in the reproductive cyclicity are most likely due to change in the activity of hypothalamo-pituitary-ovarian axis that controls normal reproductive cyclicity in mammals. Pulsatile GnRH stimulation of pituitary gonadotropes is required for and reflected in the pulsatile LH secretion [1]. However, the feedback regulation at the pituitary gland by steroids can modify or eliminate the responsiveness of the pituitary to GnRH pulses [1]. In the absence of such feedback effects, when the frequency and amplitude of GnRH pulse are fixed, the amplitude of an LH pulse is the direct measure of the releasable stores of LH in gonadotropes [29]. Due to difficulties in accessing biological samples for direct GnRH measurement, estimation of LH in circulation is regarded as bioassay for hypothamalic GnRH secretion. Exogenous administration of GnRH has been used in various animal models and human subjects to evaluate the responsiveness of the pituitary gland and regulation of gonadotropin secretion [6, 10-13, 30, 31]. In order to delineate the underlying causes for seasonal variations in the cyclicity in buffalo cows, in the present study we attempted to evaluate the response of the pituitary gland to a single injection of a range of doses of GnRH during summer and rainy months.

During summer, the minimum dose of GnRH required for eliciting a response as evident from increased circulating LH levels was 28 ng/kg BW (10 µg of GnRH bolus) and administration of this dose elicited a response similar to a physiological LH pulse in duration and amplitude (unpublished data). The response of the buffalo cows to 28 and 92.4 ng/kg BW dose of GnRH was similar to the response reported previously in prepubertal heifers administered as small repeated doses or with a bolus of GnRH [32, 33]. In the experiments conducted in the same buffalo cows during winter season however, no response to GnRH administration to 0.1, 1 and 10 µg of GnRH doses examined. On the other hand, administration of a large bolus of GnRH (i.e. 100 µg) elicited a surge-like increase in circulating LH levels similar to the response observed in cattle [24, 34]. Also, we have recently reported that 100 µg of GnRH was sufficient to induce gonadotropin surge and ovulation in buffalo cows [35]. The variation in the pituitary responsivity to exogenous GnRH administration observed during the two seasons could be due to differences in the circulating steroid milieu. It has been reported that increased circulating P_4 levels decreases the number of GnRH pulses [36] and moreover, P_4 is also known to decrease the transcription and the number of GnRH receptors on gonadotropes in cultured pituitary cells and the observation of lowest number of GnRH receptors in the pituitary membranes during the luteal phase of estrous cycle [37, 38]. Studies in ewes have established that P_4 inhibits the GnRH receptor expression by reducing the frequency of GnRH pulses [39]. It should be pointed out that the available pituitary LH stores also contribute to the magnitude of response observed during the two seasons. It is entirely possible that more releasable pool of LH was available during summer compared to rainy months.

Estradiol (E_2) is believed to be the primary factor responsible for modulating the gonadotrope responsiveness to GnRH especially around the time of occurrence of preovulatory gonadotropin surge. During the preovulatory period, E_2 causes increase in the number of GnRH receptors on membranes of pituitary gonadotropes and stimulates a sustained increase in GnRH release from hypothalamus causing LH surge and P_4 can act on the E_2 responsive cells in

hypothalamus to block the occurrence of gonadotropin surge [40, 41]. Studies on ovariectomized cattle and pony mares demonstrate that E_2 treatment is associated with an increase in pituitary responsively and circulatory LH levels [42, 43]. In ewes, basal levels of LH in the circulation are high during the follicular phase of the estrous cycle and E_2 treatment of anterior pituitary cells obtained from anestrus ewes caused an increase in LH secretion [38]. Studies in cattle by Day *et al*. [44] and Stumpf *et al*. [45] demonstrated that effects of E_2 in regulating pituitary LH secretion was independent of the seasons, however, the effects of heat stress on tonic LH as well as on GnRH-induced preovulatory surge concentrations were found to be dependent on the concentration of E_2 in plasma [46]. However, in the present study, the circulating E_2 levels during the GnRH challenge experiments were not monitored.

In response to a 100 µg dose of GnRH, a surge-like increase in the circulating LH levels was observed. Similar results in cows were observed by previous studies, where higher doses of GnRH up to 1500 µg have been administered [47] or similar doses of GnRH were administered during the periovulatory period [24, 34].

In summary, in the present study attempts were made to evaluate the seasonal changes in the pituitary responsiveness in buffalo cows. A dose of 10 µg of GnRH was sufficient to induce a pulse-like increase in circulating LH levels during summer, in the presence of lower circulating P_4 levels while, during rainy months, no increase in the circulating LH levels were observed in response to 10 µg or lower doses of GnRH indicating decreased responsiveness of pituitary in the presence of high circulating P_4 levels. Also, the dose of GnRH required for eliciting a surge-like increase in LH secretion was determined.

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