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# Quantification of kisspeptin and progesterone concentrations in ovulatory and anovulatory buffaloes

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**Abstract**
The study was conceptualized to perceive the status of the hormones progesterone (P4) and kisspeptin (Kp) was conducted throughout one complete estrous cycle in six ovulating buffaloes and similarly replicated over the period of three weeks in six anestrus buffaloes. In the ovulating buffaloes, while serum progesterone ($P_4$) concentration differed significantly and serum Kp quantification (ng/ml) did not show any significant difference, the respective concentration of both these hormones were depressed. Plausibly, though serum $P_4$ & Kp values were statistically non-significantly during the collection period, their concentration were substantially higher in the anovulatory buffaloes.

**Keywords:** Progesterone, kisspeptin, ovulating buffaloes, anestrus buffaloes

## 1. Introduction

The Asian water buffalo embodies the greatest aptitude and capability for production and reproduction. While, India has the largest number of buffaloes in the world and milk production is the backbone of the Indian dairy industry (Chakravarty, 2013) [9], there is still a tremendous scope for improving the Indian buffaloes. There is a plenty of room for increasing productivity of buffaloes through improvement of reproductive performance (Devkota, *et al.*, 2013) [11].

Progesterone plays an indispensable role in the physiological estrous cycle cascade and its escalation and plunge coincides with the corpus luteum (CL) growth and regression (Ahmad *et al.*, 1977) [1]. Progesterone concentrations fluctuate depending upon the buffalo's inherent endocrine status and is easily influenced by both environmental as well as nutritional status (Mondal *et al.*, 2007) [28] and depleted progesterone concentrations are concomitant with a surge in embryonic mortality, depleted oocytes vigour and slow endometrium growth (Diskin and Morris, 2008) [12].

It has recently been revealed that hypothalamic kisspeptin (Kp) acts upstream of GnRH and mediates sex steroid feedback and metabolic input on the reproductive axis. The kisspeptin peptide family with their receptors play a crucial role in puberty period and fertility including central activation of the HPG axis at puberty (Babiker and Shaikh 2016) [5]. This neuropeptide is required for puberty onset and maintenance of normal reproductive function, as loss-of-function mutations of kisspeptin receptor gene (KISS1R) are associated with pubertal failure, e.g. idiopathic hypogonadotropic hypogonadism (IHH) (Lents *et al.*, 2008) [21].

Despite the recent spurt of interest about this hormone, there are many uncertainties regarding its role in buffaloes. By measuring circulating Kp levels during the different phases of the estrous cycle, its fundamental physiology during the bovine estrous cycle could be comprehended and a thorough understanding of the endocrine profile of Kp during follicular dynamics would be a key step toward conceptualising kisspeptin-based therapeutic strategies. Therefore, in order to fulfil this paucity of information the experiment was conducted.

## 2. Materials and Method

### 2.1 Study site

The study was conducted at the College Instructional Livestock Farm Complex and a private farm and sample processing and laboratory work was undertaken in the Department of Veterinary Physiology, Mumbai Veterinary College, Parel, Mumbai. Sixteen apparently healthy Murrah buffaloes, maintained at private farms in Aarey Colony, Goregaon, Mumbai were taken for the present study.

### 2.2 Animals and experimental design

The buffaloes were maintained under uniform standard conditions of feeding and management with ad-libitum water. Nine postpartum lactating buffaloes were synchronized as per the standard ovsync protocol GnRH-PGF2α-GnRH (G-P-G protocol). All the buffaloes were examined by a real-time ultrasonography. Six postpartum anovulatory buffaloes were selected from private farm.

### 2.3 Blood collection

Blood was collected by jugular vein puncture and the protocol for hormonal estimations of cycling and non-cycling buffaloes differed. Blood was collected on days 0, 7, 14, and 21 day of estrous cycle for analyzing the progesterone ($P_4$) concentration in both cycling and non-cycling buffaloes. For quantification of kisspeptin, blood was collected on days 0, 3, 5, 7, 9, 11, 13, 15, 17, 19 and 21 of the estrous cycle in cycling buffaloes and on days 0, 7, 14 and 21 in anestrus buffaloes. The serum progesterone and kisspeptin concentrations were evaluated by using bovine progesterone and kisspeptin ELISA kits respectively.

### 2.4 Statistical analysis

The data was analyzed by CRD using WASP-2 (Web Agri Stat Package), ICAR.

## 3. Results and Discussion

### 3.1 Serum progesterone concentration in ovulatory buffaloes

The mean serum progesterone ($P_4$) concentrations from the day of estrus (day 0), during diestrus (days 7 & 14) and pro-estrus (day 21) of one complete estrous cycle in the investigated buffaloes are presented in Table 01.

**Table 1:** Mean ± SE for progesterone concentration in ovulatory buffaloes during estrous cycle

| Sr. No. | Days of estrous cycle | Progesterone ng/ml |
|---|---|---|
| 1. | 0 day | $0.06^{b} \pm 0.01$ |
| 2. | 7th day | $0.14^{ab} \pm 0.03$ |
| 3. | 14th day | $0.17^{a} \pm 0.03$ |
| 4. | 21st day | $0.12^{ab} \pm 0.03$ |

The statistical analysis of the data revealed that the serum $P_4$ concentration differed significantly during the complete estrous cycle. A similar trend of very low plasma progesterone concentration at estrus followed by a sharp increase during the late luteal phase, reaching a peak at Day 10-14 of the estrous cycle before declining was reported by Awasthi *et al.*, (2006) [4], Mondal *et al.*, (2007) [28], Roy and Prakash (2007) [39], Farghaly, (2012) [14], Terzano *et al.*, (2012) [43], Yilmaz *et al.*, (2014a) [45] and Kekan *et al.*, (2018) [18] but, with elevated $P_4$ concentrations when compared to ours.

Takkar *et al.*, (1982) [42] documented that the mean serum progesterone concentration in buffalo was the lowest at 0.33 ± 0.07 ng/ml at estrus. The testimonies of Noseir *et al.*, (2014) [30] and Phogat *et al.*, (2016) [34] all concur that on day of estrus the blood progesterone levels are the lowest, ranging between 0.1 to 0.5 ng/ml and this is in partial agreement with our study wherein while the $P_4$ concentrations were the lowest on the day of estrus, the concentrations were less than 0.1 ng/ml (0.06 ± 0.01 ng/ml).

In our study the $P_4$ serum concentration was the greatest (0.17 + 0.03) during the late diestrus period. Similarly, Kaur and Arora (1978) [17], Panday, (1979) [31] and Takkar *et al.*, (1982) [42] have reported mid cycle peak P4 values between day 14 to 16 of the cycle.

On the other hand, Rao and Pandey, (1982) [35] stated that $P_4$ plasma levels reached maximum values (1.72 to 3.11 ng/ml) on Day 18, Jainudeen, (1986) [16] maintained that they peaked (2.4 ng/ml) by Day 8, Kumud, (1999) [20] disclosed maximum $P_4$ (2.74 ± 1.85 ng/ml) on Day 9 of the cycle and Noseir *et al.*, (2014) [30] recorded the highest progesterone concentration (8.56 ± 0.07 ng/mL) on Day 16 of the cycle in buffaloes.

Ronchi *et al.*, (2001) [38] and Terzano *et al.*, (2012) [43] postulated that the $P_4$ concentrations are also governed by nutritional and environmental variances and the suboptimal feed teamed with soaring environmental temperatures might be accountable for long anestrus in buffaloes as observed in our study. Further, Sharma (2003) [40], Ahmed (2006) [2] and Ahmed *et al.*, (2006) [2] proposed that ovarian dormancy is the foremost reproductive disorder in buffalo reproduction, particularly noted in animals kept in small holder farms and subjected to a lot of stressful circumstances such as malnourishment, parasitism, deficient sanitation and pollution which again resembles the circumstances of buffaloes incorporated in our assessment.

### 3.2 Serum progesterone concentration in anovulatory buffaloes

The mean serum progesterone concentrations from the day of first collection (day 1) and thereafter weekly in the reviewed anestrus buffaloes are presented in Table 02.

**Table 2:** Mean ± SE for progesterone concentration in anovulatory buffaloes

| Sr. No. | Days of estrous cycle | Progesterone ng/ml |
|---|---|---|
| 1. | 0 day | 0.45 ± 0.06 |
| 2. | 7th day | 0.46 ± 0.06 |
| 3. | 14th day | 0.55 ± 0.04 |
| 4. | 21st day | 0.53 ± 0.07 |

The statistical analysis of the data revealed that the serum $P_4$ concentration differed non-significantly during the collection period, but the serum $P_4$ values appeared to be substantially elevated in the anovulatory buffaloes.

El-Wishy (2007) [13] while recommending progesterone monitoring as an independent and precise method for assessment of the reproductive potential in postpartum buffaloes elucidated upon the four main forms of anestrus i.e., true anestrus (inactive ovaries and small and medium sized anovulatory follicles), sub estrus (unobserved and silent heat), prolonged luteal activity & ovarian cysts and lastly pregnancy. Mondal *et al.*, (2010) [29], considering the $P_4$ levels regression, near the cycle culmination and a sharp ascent during the luteal phase, advocated that the CL function can be monitored by progesterone determination in both cattle and buffaloes who present overt and silent estrus.

Farghaly, (2012) [14] affirmed this based on his work on Egyptian buffaloes wherein he registered that those having serum progesterone concentrations ≥1.0 ng/ml in at least one of the two samplings carried out during his exploration were considered cycling, and those with both samples being 1.0 ng/ml or more were considered as anovulatory/anestrous buffaloes. In our current study also, we observed an analogous proclivity of serum progesterone values being higher in anestrus buffaloes. Conversely Peter *et al.*, (2009) [33] defined true anestrus as lack of ovarian progesterone

production.
Mondal and Prakash, (2002) [27] ascertained that in buffaloes exhibiting silent estrus, the $P_4$ concentrations amplified from 0.38 ± 0.02 ng/ml during periestrus phase (day-1 to day 1) to 0.51 ± 0.07 ng/ml during early luteal phase (day 2 to day 5) and then advanced to 1.30 ± 0.13 ng/ml during mid-luteal phase (day 6 to day 14) followed by a reduction to 0.66 ± 0.13 ng/ml during late luteal phase (day-4 to day -2) and while the $P_4$ concentrations in the studied buffaloes were lower, a resembling trend was perceived.
Perera, (2011) [32] stipulated that buffalo having $P_4$ concentrations above 3 nmol/l (0.94 ng/ml) denoted the presence of luteal function, those with $P_4$ concentrations below 1 nmol/l (0.31 ng/ml) signified the absence of luteal function and those having intermediate values being were considered inconclusive and since the scores of our assessment lie between the two margins we can conclude that the buffaloes included were not in true anestrus.

### 3.3 Serum kisspeptin concentration in ovulatory buffaloes

The mean serum kisspeptin (Kp) concentration (ng/ml) during estrus (day 0), metestrus (day 3), di-estrus (days 5, 7, 9, 11, 13, 15, 17) and pro-estrus (days 19 & 21) of one complete estrous cycle in the investigated buffaloes are presented in Table 03.

**Table 3:** Mean + SE for kisspeptin concentration in ovulatory buffaloes during estrous cycle

| Sr. No. | Days of estrous cycle | Progesterone ng/ml |
|---|---|---|
| 1. | 0 Day | 0.08 ± 0.00 |
| 2. | 3 Day | 0.10 ± 0.01 |
| 3. | 5 Day | 0.10 ± 0.01 |
| 4. | 7 Day | 0.10 ± 0.01 |
| 5. | 9 Day | 0.10 ± 0.00 |
| 6. | 11 Day | 0.11 ± 0.01 |
| 7. | 13 Day | 0.10 ± 0.01 |
| 8. | 15 Day | 0.10 ± 0.01 |
| 9. | 17 Day | 0.10 ± 0.00 |
| 10. | 19 Day | 0.12 ± 0.01 |
| 11. | 21 Day | 0.12 ± 0.01 |

The serum kisspeptin concentration did not show any significant difference during the scrutinized estrous cycle as revealed by the statistical analysis of the data and looking at the values, besides the slight low on day 0 (0.08 ± 0.00) and a little rise on days 19 & 21 (0.12 ± 0.01), the serum kisspeptin concentration was consistent (0.10 ± 0.01) throughout the cycle. These inferences are in close agreement with earlier reports Rizzo *et al.*, (2018a) [37] who reported 0.10 ng/ml kisspeptin concentration in healthy dairy cows on estrus day.
In juxtapose to our findings, the reports of Mondal *et al.*, (2015) [26] reported a steady decline trend from the day of estrus to day 4 after which they witnessed a sharp rise which peaked on day 6 of the cycle, following which the concentration then depleted from day 7 to touch the baseline on day 10 which then displayed an upward progression to peak on day 12 of the cycle.
Rizzo *et al.*, (2018b) estimated the circulating kisspeptin concentrations in dairy cows on 10, 12, 14 and 16 days after calving and found them to be 0.15, 0.11, 0.15 and 0.12 (ng/ml) respectively.
Intriguingly, Mondal *et al.*, (2015) [26] in cycling non-lactating cows and Kumar *et al.*, (2021) [19] in cycling Bengal does recorded an average of three and four kisspeptin peaks respectively with varying amplitudes during the entire estrous cycle which was not observed in our study using buffaloes.
Mondal *et al.*, (2021) [25], associating plasma kisspeptin with follicular emergence recorded three kisspeptin peaks with varying amplitudes, the first peak having the highest amplitude appeared a day before the preovulatory LH surge, the second and third surges were recorded on day 6 and 12 of the estrous cycle, respectively and these peaks corresponded to the emergence of follicular waves.
Accordingly in our study we have obtained the maximum number of antral follicles during the mid-luteal stage and the Kp concentration also showed a slight rise during that period and similarly the high follicle count during the follicular phase can be ascribed to the Kp perk during that period. Further, the role of Kp on the growth of dominant follicles can be envisualised by the computation of maximum number of large follicles during the follicular phase when the Kp concentration was also at its highest. We could therefore correlate the dynamicity of plasma kisspeptin with follicular emergence during the studied estrous cycle.

### 3.4 Serum kisspeptin concentration in anovulatory buffaloes

The mean serum kisspeptin concentrations (ng/ml) on day 1, day 7, day 14 and day 21 collected from anovulatory investigated buffaloes are presented in Table 04.

**Table 4:** Mean + SE for kisspeptin concentration in anovulatory buffaloes during anestrous cycle

| Sr. No. | Days of estrous cycle | P4 ng/ml |
|---|---|---|
| 1. | 0 day | 0.12 ± 0.01 |
| 2. | 7th day | 0.63 ± 0.24 |
| 3. | 14th day | 0.51 ± 0.25 |
| 4. | 21st day | 0.61 ± 0.24 |

The serum kisspeptin concentration did not show any significant difference during the three weeks collection period as conveyed by the statistical analysis of the data, nevertheless, there was a small spike on day 7 and then a very nominal dip followed by a slight rise. Understandably, the serum kisspeptin concentrations were numerically much higher in the anovulatory buffaloes evaluated.
Rizzo *et al.*, (2018a) [37] measured Kp levels in cows having ovarian follicular cysts as 0.13 ng/ml kisspeptin and have proposed that significantly higher blood Kp concentrations are detected in cows with follicular cysts as compared to healthy cows (0.09 ng/ml). They reasoned that loftier blood Kp concentrations in cows with ovarian follicular cysts when compared to healthy cows imply that the hypothalamic nuclei associated with Kp secretion may be stimulated by greater steroid levels.
In seasonal breeders like buffalo cows, mares, ewe and does, the chief cause of post pubertal infertility is seasonal anestrus. Kisspeptin plays an essential role in receiving stimulatory estrogen signals and generating the full positive feedback GnRH/LH surge. Intravenous administration of kisspeptin therefore stimulated an LH surge in seasonally anestrus ewes (Smith *et al.*, 2006, Caraty *et al.*, 2007, Caraty *et al.*, 2012, Li *et al.*, 2012 and Foster and Hileman, 2015) [6, 8, 7, 22, 15] does (Decourt *et al.*, 2019) [10] and mares (Briant *et al.*, 2016 and McGrath *et al.*, 2016) [6, 24] though ovulation was only seen in pony mares, and this is now incorporated as a management tool in the hormonal combination used for the estrus synchronization in bovines (Macedo *et al.*, 2014 and Mondal *et al.*, 2015) [23, 26]. Further, kisspeptin plays a regulatory role

in equine cyclicity and seasonality, but its effects in long-day breeders like the mare differ from those in short-day breeders like the ewe (Wilborn, 2008) [44] which could explain its reasonable success in buffaloes.

### 4. Conclusion
In our exploration, while significantly different progesterone concentrations were perceived in ovulatory buffaloes during one estrous cycle, these were non-significant in the anovulatory buffaloes. Further, our investigation of the kisspeptin concentrations showed non-significance in ovulatory buffaloes during the assessed complete estrous cycle as well as in the anovulatory buffaloes three-week sampling.

The hormone kisspeptin stimulates the HPG axis causing autocrine and paracrine actions in the ovaries and it has also been suggested that this neuropeptide could be an indispensable tool in the restoration of reproductive cycle in anestrus animals. While there is much literature on the use of Kp in different species to enhance reproductive capabilities, there is hardly any publication on the actual values of then hormone during the cycle or in non-cycling animals. We can conclude that there is substantial potential for using kisspeptin in the restoration of ovarian cyclicity in anestrous buffaloes. Undeniably, there is need for many more studies on larger buffalo populations for understanding of its exact role in the reproductive physiology of our nation's key livestock species.

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