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Evaluation of Hormonal and Biochemical Profile of Ladakhi Cow during Estrous Cycle: The Future Perspective of Reproductive Management of Ladakhi Cow

Preeti Kumari[1,2], Yogesh Singh[1,3], Vijay K. Bharti[1*] and Indu Sharma[2]

[1]DRDO-Defence Institute of High Altitude Research (DIHAR), Leh, Ladakh – 194101, Jammu and Kashmir, India; vijaykbharti@rediffmail.com
[2]Department of Zoology, Panjab University, Chandigarh – 160014, India
[3]ICMR-Regional Medical Research Centre, Port Blair – 744103, Andaman and Nicobar Islands, India

Abstract

The Ladakhi cow is an indigenous breed from Ladakh, India, and has a unique genetic makeup to adapt to high-altitude hypobaric stress. Furthermore, little study is available on examining the Ladakhi cow's blood biochemicals and reproductive hormonal status during various stages of the estrous cycle. Hence, the current study was conducted on eight reproductively mature Ladakhi cows at the experimental dairy cattle unit in Leh-Ladakh, India, to determine the changes in serum biochemical and hormonal levels during the estrous cycle. The estrous behaviour was initially observed before the blood sampling on days 0 (the day the cow exhibited heat symptoms), 2nd, 8th, 17th, and 21st days. The serum was isolated for biochemical and hormonal profiling as per standard procedures using commercially available kits. The results revealed that serum biochemicals, viz. Ca, Fe, ALT, AST, ALP, TG, TP, Albumin, Glucose, UA, Creatinine, and Urea levels were within the normal range of cows at high altitudes. However, reproductive hormones, e.g. GnRH, FSH, LH, progesterone, estrogen, PGF2α, IGF-1, and Insulin varied from the reference level at different stages of the estrous cycle. This study's findings have provided new insights into the serum biochemical and hormonal profiling of Ladakhi cows during the estrous cycle and this information may help to develop a reference range and baseline value for the serum biochemicals and hormonal levels of Ladakhi cows for other breeding programs and health monitoring.

Keywords: Biochemical, Estrus Cycle, High-Altitude, Hormonal, Ladakhi Cow

1. Introduction

The Ladakh is located at 3,500 m above MSL and accompanied by extremely harsh climatic conditions such as temperatures (i.e., +35°C to -35°C), high UV index (7-11), low snowfall, wind, low precipitation, low relative humidity, intense sunlight, etc. These environmental factors adversely impact the health, production, and reproduction of various species of domestic animals, including cattle[1]. Additionally, it is a popular tourist destination with significant strategic importance for national security and the increasing local population. As a result, there is a growing demand for fresh animal-origin food on livestock; additionally, the high-altitude environment leads to cardio-respiratory, endocrine, metabolic, nutritional, and thermal strain in animals and humans[2,3]. Therefore, to combat these adverse effects of high altitude stress on humans, fresh food with high nutrition levels is required to overcome the negative effects.

The harsh, arid climate makes it challenging to raise livestock because there is a lack of fodder and water, which causes nutritional stress. However, due to natural selection, all populations of native livestock have evolved to be able to withstand the pressures of high altitude and food scarcity. Generally, local farmers rear local Ladakhi cattle, goats, and yak for milk and their by-products; Ladakhi cattle are a small, native breed of Ladakh, with a population of 3417 as of the 19th Livestock Census and have a unique genetic makeup and a remarkable capacity to adapt to high-altitude hypobaric stress[3,4]. In addition, the Government of India has recognized Ladakhi cattle as the 42nd native breed of India.

*Author for correspondence

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A few studies have examined the impact of high altitude stress on Ladakhi cattle. For instance, haematological parameters can be used to assess how well animals have adapted to their environment[3,5]. Besides, biochemical parameters are responsible for various body functions, and their deficiency results in the impairment of functions and induces structural and physiological abnormalities[6]. According to Kumar *et al.*[5] and Ahmed *et al.*[7], changes in the biochemical and haematological constituents are crucial indicators of the animal's physiological or pathological state. Therefore, in high-altitude areas, Ladakhi cattle health requires a clinical examination, as well as various biochemical assays and haematology examinations, which aid in the diagnosis or may reveal the type and severity of the disease.

Additionally, Ladakhi and Jersey crossbred cattle raised at high altitudes showed a significant variation in haematology parameters, according to studies by Bharti *et al.*[2] and Giri *et al.*[3]. Although they claimed that these parameters varied within the published normal reference range for cattle, there was no reference or normal range of these parameters for cattle raised at high altitudes to compare. Furthermore, hormones regulate homeostasis and enhance essential traits in cattle, including fertility, growth and development, and health[8]. In light of this, research into hepatic metabolism and hypothalamus-pituitary-gonad functioning is crucial for regular physiological and behavioural changes associated with estrous in reproduction and is necessary for breed improvement. Thus, the present work was conducted to investigate the Ladakhi cow's blood biochemical profile and hormonal profile during the estrous cycle.

2. Methods and Materials

2.1 Experimental Design

The present study was carried out under guidelines approved by the Institute Animal Ethical Committee at the dairy cattle unit of DIHAR-DRDO, Leh, India. For the study, a total of 8 (eight) reproductively mature Ladakhi cows were used. The management of cow housing was a semi-intensive system, and according to standards, cows were fed with feed concentrate and dry alfalfa grass, and water was available at all times. Cows were observed twice daily, in the morning and evening, for visible signs of heat or estrous. Consequently, cow blood samples were taken starting the 0^{th} day after estrous symptoms, then on the 2^{nd}, 8^{th}, 17^{th}, and 21^{st} days. The serum was isolated and used for biochemical and hormonal analysis as described below.

2.2 Analysis of Serum Biochemical and Hormonal Parameters

Biochemical analysis, i.e., Calcium (Ca), Iron (Fe), Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), Alkaline Phosphatase (ALP), Triglyceride (TG), Total Protein (TP), Albumin, Glucose, Uric Acid (UA), Creatinine and Urea, was conducted by using commercially available kits (Mindray, Shenzhen, China) in fully automated biochemistry analyzer (Mindray BS-120). Hormonal analysis was done using commercially available ELISA kits (BT LAB – Bioassay Technology Laboratory, Shanghai, China), i.e., Gonadotropin-Releasing Hormone (GnRH), Follicle-stimulating hormone (FSH), Luteinizing Hormone (LH), progesterone, estrogen, Prostaglandin ($PGF_{2\alpha}$), IGF-1 (Insulin-like Growth factor-1) and Insulin, and the reading was taken in a Multi-Mode Microplate Reader (i3x SpectraMax, Molecular Devices, USA).

2.3 Statistical Analysis

The SPSS software was used to analyze all the data, and values are presented as means and standard errors (SE) with a $p<0.05$ as the level of significance.

3. Results

The cows were observed for heat symptoms during the morning and evening when roaming free during the daytime. The cow displayed heat symptoms, including mucus discharge, mounting on another cow, restlessness, swelling and reddening of the vulva, etc. Heat symptoms are generally very fleeting, so careful observation is required. The estrogen hormone causes numerous internal and external changes to reciprocate the conception process and has impacted heat symptoms. Many internal changes were observed during the estrous cycle, out of which a few parameters were studied in this research.

3.1 Biochemical Changes during the Estrous Cycle

Considering the estrous cycle stages, we have estimated various biochemical parameters during the estrous cycle of the Ladakhi cow and the results are shown in graphs.

As seen in Figure 1, Ca showed no significant difference during an estrous cycle; P showed a low value on the 8th day of the estrous cycle but the difference was insignificant; whereas, Fe showed variation during the cycle with a high level on the 2nd day, but the difference was negligible compared to others.

As seen in Figure 2, ALT and ALP activities showed no significant difference within the estrous cycle, but AST activity was significantly ($p<0.05$) high on the 21st day. TG was high on the 21st and 0th days, but the difference was insignificant within the cycle (Figure 2).

TP showed no considerable variation within the estrous cycle, whereas albumin showed a low value on 2nd day of the estrous cycle but is insignificant as compared to others (Figure 3). In Figure 3, glucose shows no variation through the estrous cycle.

UA in Figure 4 showed a significantly ($p<0.05$) low value on the 21st day; on the 17th day also it showed a significant ($p<0.05$) low value as compared to other days. Further, in Figure 4, creatinine showed no significant difference within the estrous cycle, whereas urea showed a significantly ($p<0.05$) high value on the 0th day of the estrous cycle.

3.2 Hormonal Fluctuation during the Estrous Cycle

In Figure 5, LH shows a significantly ($p<0.05$) high value on the 0th day, referred to as LH surge, and gradually decreased. FSH level fluctuated during the cycle and was significantly high on the 0th day, whereas GnRH level within the anestrous cycle was insignificant.

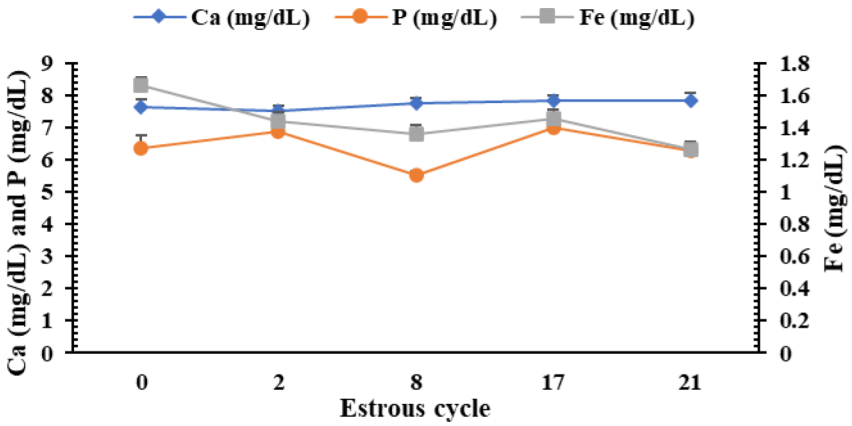


Figure 1. Variation in Ca, P, and Fe during the estrous cycle of Ladakhi cow.

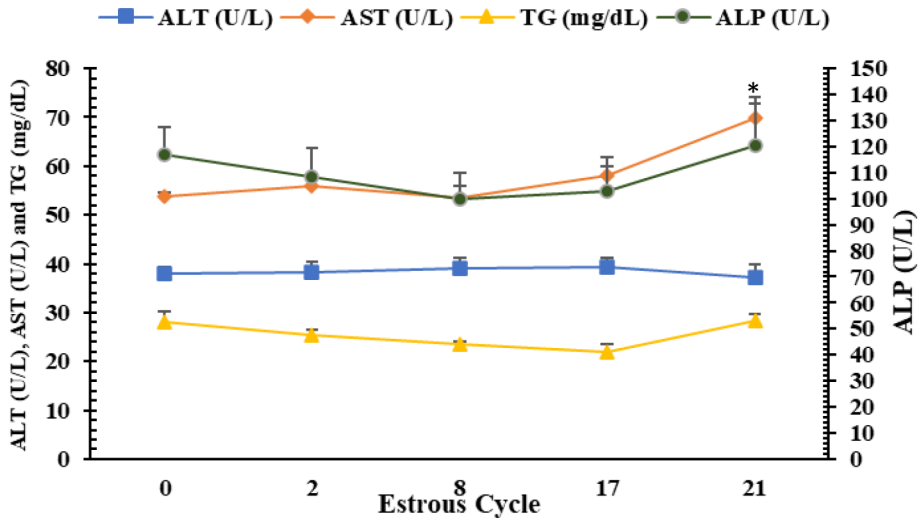


Figure 2. Variation in ALT, AST, TG, and ALP activities during the estrous cycle of Ladakhi cow.

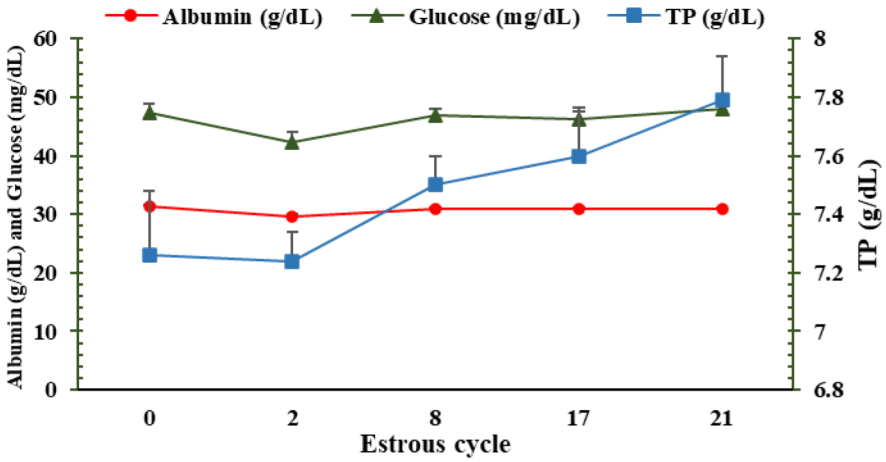


Figure 3. Variation in TP, Albumin, and Glucose during estrous cycle of Ladakhi cow.

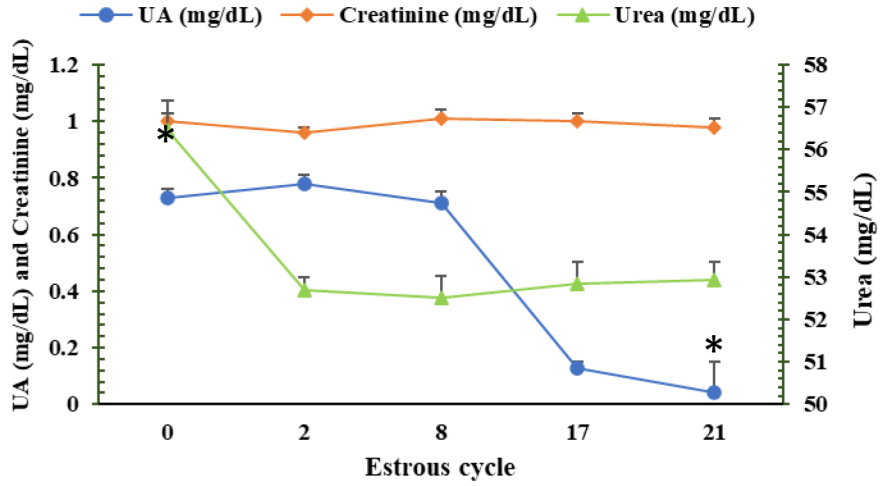


Figure 4. Variation in UA, creatinine, and urea during the estrous cycle of Ladakhi cow.

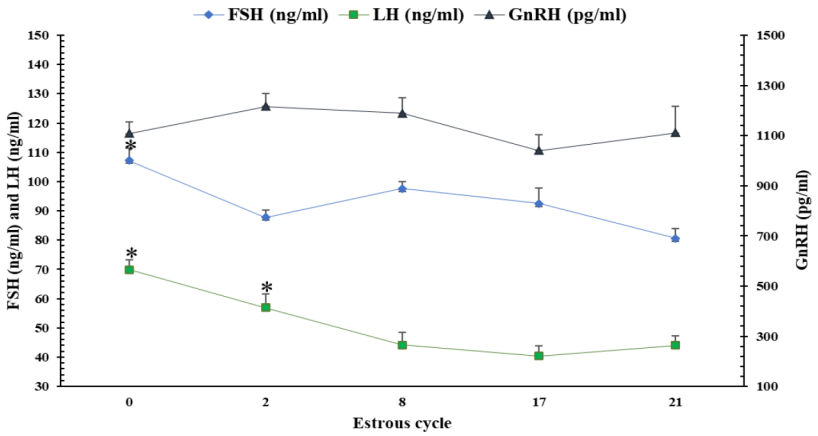


Figure 5. Variation in GnRH, FSH, and LH levels during the estrous cycle.

As seen in Figure 6, the estrogen level was significantly high on the 21st and 0th day, which induces estrous symptoms. However, progesterone level gradually increased significantly ($p<0.05$) to the highest level on the 17th day. At the same time, the $PGF_{2\alpha}$ level does not show any significant change during the estrous cycle.

In Figure 7, IGF-1 gradually increased from the 0th day to the 21st day of the estrous cycle, and it showed a significantly ($p<0.05$) high value on the 21st day, whereas the insulin level increased till the 0th day and almost no change to 17th day and increased to 21st day of the estrous cycle but the change was not significant.

4. Discussion

The cow undergoes several changes during the estrous cycle that are necessary for reproduction, viz., the proestrus, estrous, metestrus, and diestrus stages of the estrus cycle, which lasted 17–21 days, characterized by changes in the uterus, ovary, and hormone levels. The estrus (heat) lasted 6 to 30 hours, and the metestrus lasted 3 to 5 days when ovulation would occur. Diestrus lasted about 13 days, and proestrus lasted 2–3 days, preparing the body for estrus.

A few essential minerals, namely Ca, P, and Fe, exhibit slight variations, which are not significant during the different days of the estrous cycle and they have an important role in reproductive functions in cows. Ca plays a critical role in the gonadotropic regulation of ovarian steroidogenesis[9], and a slight phosphorus deficiency can disrupt the pituitary-ovarian axis and interfere with ovulation[10]. Low serum iron levels (Fe) have been shown to affect regular gonadal activity, possibly impairing hormonal output and negatively impacting overall physiological function[11]. In cattle and many other animals, activities of the liver enzymes, ALT, AST, and ALP, are routinely measured in serum and used as components of accurate hepatic diagnostics[12]. In our study, ALT and ALP activities do not change from the 0th to the 21st day, while AST activity is noticeably higher on

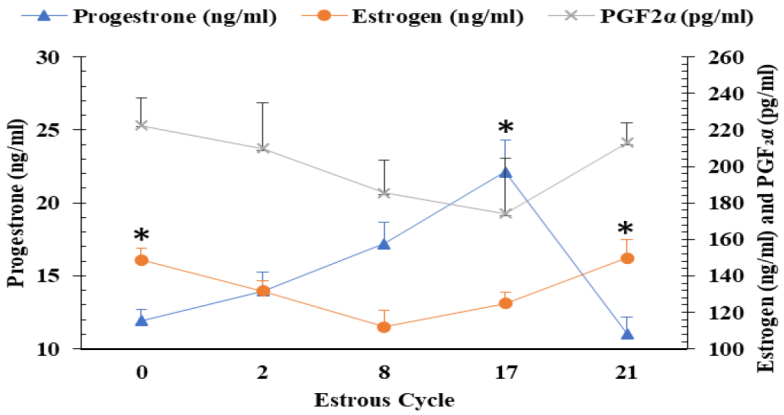


Figure 6. Variation in estrogen, progesterone, and $PGF_{2\alpha}$ levels during the estrous cycle.

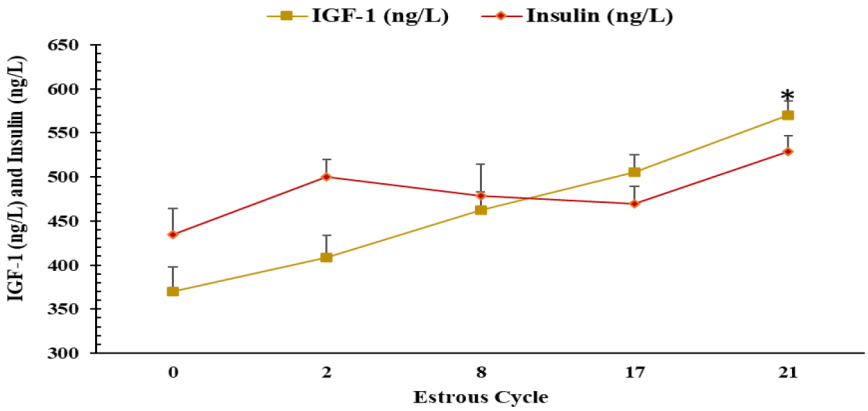


Figure 7. Variation in IGF-1 and insulin levels during the estrous cycle.

the 21st day (Figure 2). Serum AST activity level increased ($p<0.05$) before the onset of estrus symptoms[14] due to heightened metabolic activity. AST, ALT, and ALP effects on the estrus cycle are unknown. TG represents the liver health of the cattle, although it indicates changes in the estrous cycle but not significantly (Figure 2). However, the TG level in Ladakhi cows is high from the reference level (0-14.0 mg/dL) due to the high altitude adaptations of cattle[4]. TG is a good source of energy substrate for ATP generation under hypoxic and cold conditions, which is a metabolic adaptation mechanism in high-altitude animals.

The liver produces albumin and regulates the osmotic pressure in the bloodstream. Reduced albumin levels have been linked to malnourishment, inflammatory disorders, liver disease, and renal disease[14]. Although albumin levels within the reference range[4] are not known to affect a cow's estrous cycle, Ladakhi cows' albumin levels do not change during the estrous cycle (Figure 3). Dairy cow immunological status has been assessed using TP as a possible marker[15]. The variation in blood serum proteins (albumin, globulin, total protein, and albumin-to-globulin ratio) in dairy cattle should be interpreted as indicators of cow's health only after taking into account breed, environmental, and individual cow factors (parity and lactation stage)[16]. Total protein level varies during the Ladakhi cow's estrous cycle; however, this variation is not statistically significant (Figure 3). The TP influence on the estrous cycle, however, is unknown. Blood glucose is an essential component for farm animals to keep the estrous cycles going. The body's physiological activities require a minimum of 60–100 mg/dL of blood glucose[17]; nevertheless, low blood sugar and infertility may be linked[18]. In Figure 3, glucose shows no significant change throughout the estrous cycle, representing the normal estrous cycle in Ladakhi cows.

Endogenous protein catabolism may be assessed using blood creatinine and uric acid generation[19]. Uric acid levels are considerably low ($p<0.05$) on days 17th and 21st of the estrous cycle (Figure 4). This might be attributed to protein catabolism, but no other credible explanation has been uncovered however, the creatinine level does not alter through the estrous cycle (Figure 4). Serum urea is the most common metabolite used to measure dairy cows' protein status and consumption[20]. Ladakhi cows had significantly higher blood urea levels ($p<0.05$) on the first day of the estrous cycle (Figure 4). In contrast, the urea level in Ladakhi cows is higher than the reference level due to hypoxia at high altitudes[4]. Several investigations have shown that increasing urea from high-protein meals can directly reduce fertility by altering progesterone in micro-uterine circumstances[21-24]. High urea levels may interfere with hormone regulation during the estrous cycle. Serum biochemicals do not directly affect the estrus cycle; further research is required.

The hormones operate using positive and negative feedback mechanisms to regulate the estrous cycle of cattle[25]. The estrous cycle is regulated by GnRH, FSH, LH, progesterone, estrogen and PGF2α. GnRH is consistently released in minimal quantities from the tonic centre of the hypothalamus. It is carried to the pituitary gland through the hypophyseal portal bloodstream, and it stimulates the release of FSH and LH[26,27]. Heifers and cows exhibit sexual receptivity during behavioural estrus and will assume a standing position to be mounted. The fast growth of the dominant follicle (DF) before ovulation leads to a drop in progesterone levels[28]. The preovulatory surge of GnRH triggers a simultaneous surge of LH and FSH. Ovulation occurs when blood progesterone levels are low, and LH pulse frequency increases to one per hour for two to three days[25,29]. The luteal phase of the estrous cycle begins after ovulation and it occurs 10 to 14 hours after estrous. This phase referred to as met-estrous, is marked by the development of the corpus luteum (CL) from the collapsed ovulated follicle (corpus haemorragicum) and usually lasts 3–4 days[30]. After ovulation, the CL develops when the granulosa and theca cells of the ovulated DF undergo luteinization and begin producing progesterone. This spike in progesterone level is essential for preparing for pregnancy or initiating a new estrous cycle[31]. Progesterone levels are elevated during the di-estrous period, stimulating the anterior pituitary gland to produce FSH, initiating several cycles of follicle formation. Progesterone concentrations fall during the pro-estrous stage when the corpus luteum regresses in response to prostaglandin F released from the uterus[32]. The baseline level of plasma LH is low and LH pulses are rarely produced during the luteal phase of the estrous cycle. LH regulates luteal function during gestation and the estrous cycle in cattle[33-36].

The LH in Figure 5 exhibits a significant ($p<0.05$) increase on day 0, this is known as the LH surge. It then gradually declines. The LH surge causes the rupture of the dominant ovarian follicle, approximately 24 to 32 hours later, thus releasing the egg. Friesland and Brangus cows had LH levels up to 60.0 ng/mL[37], whereas Ladakhi

cows had even higher levels, 69.69 ng/mL. LH level varies among the cow breeds- 119-139 ng/mL in crossbred (Hereford X Angus X Simmental) cows[38], 2.5 to 4.6 ng/mL LH at the peak in the Holstein breed[39], and Mithun breed shows 15 ng/mL LH at peak[40]. These variations may be due to the breeds or environmental factors, whereas hormone levels may vary among the breeds although the patterns of hormonal release remain the same. The onset of estrus and preovulatory surges of LH and FSH have been observed in cattle[41,42]. The LH and FSH surges before ovulation trigger several physiological and morphological changes in the ovarian follicle, resulting in its maturity and rupture to release a fertilizable egg[43]. FSH levels fluctuate throughout the estrous cycle of the Ladakhi cow, but are exceptionally high on day 0; FSH is secreted during and shortly after estrus and stimulates the growth of a fresh wave of ovarian follicles. FSH levels in the Sahiwal breed vary from 1.58 to 1.69 ng/mL during the estrous cycle, while in Holstein cows, the levels fluctuate between 14.0 and 121 ng/mL[44]. A similar tendency is evident in Ladakhi cows, although the FSH level is 80 to 107 to 80 ng/mm. GnRH release throughout the estrous cycle does not show any significant change; it fluctuates throughout the cycle. The pulsatile secretion of GnRH into the pituitary's portal circulation by the hypothalamus regulates the release of gonadotropins[45-47]. Improvement of conception following GnRH treatment during estrus has been attributed to preventing ovulation failure or reducing variation in the interval to ovulation[48,49].

In Figure 6, a low estrogen level is seen on the eighth day, gradually rises until the twenty-first day, and then gradually falls from the first to the eighth day; the significantly high estrogen levels on the twenty-first and first days account for the cow's induced estrus symptoms. During the estrous cycle, the estrogen levels in Ladakhi cows range from 112.16±8.70 to 149.88±10.18 ng/mL. Mithun cows have lower estrogen levels compared to Ladakhi cows, yet the highest oestrogen level of 8.3±0.3 pg/mL is reached before estrus[50]. Mondal *et al.*[51] reported that the mean highest peak concentration of estrogen was 45.69±2.32 pg/mL before the onset of estrus in Mithun cows. Likewise, Punganur cows' estrogen level was high near estrus, 22.25±1.17 pg/mL[52], suggesting that Ladakhi cow estrogen levels are higher than average but their hormonal tendencies are like those of other breeds. The progesterone level increases gradually from day 0 to day 17, reaching a significantly high level ($p>0.05$), which induces endometrial alterations and prepares cows for pregnancy. During the estrous cycle, Ladakhi cows have progesterone levels ranging from 11.04±1.13 to 22.12±2.20 ng/mL, with the value on day 17 being the highest and on day 0 being the lowest. Progesterone levels in mixed breeds (Jersey, Friesian, Jersey X Friesian) peaked at 6-7 ng/mL around 15-18 days of the cycle before decreasing. The progesterone level of Mithun cows reached a peak progesterone level of 3.7 ng/ml on the 15th to 17th day of the estrous cycle. Punganur cows had a mean progesterone level of 10.67±0.89 ng/mL on the 15th day of the estrous cycle[52]. Although progesterone suppresses cows' estrus behaviour, a minimum progesterone level is necessary to express estrus[53]. In contrast, the $PGF_{2\alpha}$ level presented in Figure 6, shows no discernible change throughout the estrous cycle of Ladakhi cow which ranges from 174.11±30.24 to 222.57±15.06 pg/m. $PGF_{2\alpha}$ induces luteolytic changes in the body, leading to a decrease in progesterone levels and is essential for the onset of the subsequent estrous cycle[54].

Follicular growth is mainly governed by gonadotropins from the pituitary gland and influenced by the synthesis of local and systemic growth factors like IGF-1. The IGF system is mostly present in the liver and reproductive organs and promotes positive effects on embryonic development[55,56]. On the other hand reduced IGF-1 secretion, caused by negative energy balance (NEB), alters ovarian follicular estradiol production, thereby suppressing the expression of estrus[57,58]. Insulin is now understood to be a crucial circulating signal that mediates energy. There is compelling evidence that insulin regulates reproduction and may act as a key signal bridging metabolism and reproductive status. Studies conducted *in vitro* showed that follicular development, steroidogenesis, oocyte maturation, and subsequent embryo development are all mediated by insulin and IGF-1 (homeostasis, as first proposed by Banting *et al.*[59]. Maintaining peripheral glucose homeostasis through stimulation of glucose uptake, oxidation, and storage is insulin's primary function[60,61]. In Figure 7, IGF-1 level in Ladakhi cows increased significantly ($p>0.05$) from 370.45±27.73 to 569.98±15.86 ng/L between the 0th and 21st day of the estrous cycle. Likewise, the mean value of IGF-1 concentration in the Sahiwal breed cow continued to rise steadily up to the 21st day of the estrous cycle, measuring 125.81±4.87 ng/mL[62]. Insulin levels vary from 434.33±29.94 to 528.72±17.63 ng/mL during the estrous cycle of Ladakhi cow, with the peak value occurring on the 21st day. Similarly, in the Sahiwal breed cow, the

insulin level is high on the 21st day, i.e., 46.11 ± 1.57 µ IU/mL[62]. The increase in insulin and IGF-1 levels had a stimulatory effect on follicular growth (small follicles) before superovulation[63]. Insulin regulates CL function through glucose availability and hormone production in cattle[64].

5. Limitations of the Study

In our study, a varying level of hormone was observed, which could be due to the differences in the sampling seasons and climate at the high altitude, the animal's age and their physiological status. Furthermore, the current study was conducted only with 8 Ladakhi cows; future research may be carried out with a larger sample size.

6. Conclusion

In conclusion, the present study was carried out to investigate the Ladakhi cow's serum biochemical and hormonal profiles during the estrous cycle, which indicated that calcium, iron, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, albumin, glucose, uric acid, and creatinine were within the normal range. However, triglycerides, total protein, and urea are higher than the reference range of high-altitude adaptation. Many reports on estrous cycle hormones of different cow breeds diverged from Ladakhi cows, but the release pattern is almost similar, indicating normal estrous cycle regulation. This study added a new dimension to our understanding of the Ladakhi cow's reproductive physiology, and these findings will be helpful for veterinary physicians in managing health and reproduction in the extreme climate of high altitude.

7. Acknowledgment

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