

# Serum Anti-Müllerian Hormone and Cytokine Profiling of Bubalus bubalis (Murrah buffalo) Calves for Puberty Prediction

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**Abstract:** The present study incorporated ten buffalo calves aged 0 to 6 months, with an average weight of 35 kg, and ten buffalo heifers aged 12 months, with an average weight of 200 kg, to study the cytokine and AMH profile in relation to pubertal advancement. Venous blood samples (5ml) were collected from buffalo calves on the day of birth (day 0), day 15, day 30, day 60, day 90, day 120, day 150, and day 180. A single blood sample was collected from Buffalo heifers (1 year age). Cytokines: IFN- $\gamma$ , IL-6, IL-1, IL-13, TNF- $\alpha$ , and TGF- $\beta$ , and anti-Müllerian hormone: AMH were estimated using respective ELISA kits. At birth, cytokine levels in serum showed a varied pattern, with lower levels of IFN- $\gamma$ , IL-6, and IL-13, whereas IL-1, TNF- $\alpha$ , and TGF- $\beta$  were higher. Throughout the study, IFN- $\gamma$ , IL-13, and TGF- $\beta$  levels remained relatively stable, whereas IL-1, IL-6, and TNF- $\alpha$  increased notably by day 180. IL-1, TNF- $\alpha$ , and IL-6 levels were higher ( $P < 0.01$ ) from birth to 180 days as well as on day 365. AMH levels remained consistent from birth to 180 days, indicating a marked increase at Day 15 ( $33.49 \pm 12.63$  ng/L), followed by a decline to  $4.60 \pm 1.55$  ng/L at the end of the first year. Implications of the Karl Pearson correlation coefficient revealed a negative correlation between AMH levels and IFN- $\gamma$  and TNF- $\alpha$ . AMH was positively correlated with IL 13 and TGF- $\beta$ . Hence, it was concluded that IFN- $\gamma$  and TNF- $\alpha$  are predictive markers for a reduction in AMH levels and hence, setting up puberty in buffalo heifers.

**Keywords:** Cytokines, puberty, ELISA, buffalo, heifers, Karl Pearson correlation.

## INTRODUCTION

Puberty, initial stage of maturing growth for reproduction, essential for future reproduction, involves the coordinated development of the endocrine and reproductive systems. Various factors, including age, species, genetic makeup, weight, growth, cytokines (immune molecules), and energy levels, play a part in determining when puberty begins [1].

Buffaloes have delayed puberty, higher age at first calving, a propensity for seasonal breeding, and silent heat. A major concern for buffalo breeders and veterinarians is ensuring that the buffaloes conceive at the right time and the age at which puberty begins so that breeding can occur at the appropriate time. Prepubertal anestrus significantly impacts an animal's lifespan productivity due to the delay in the period between the first calving and lactation.

In buffalo heifers, puberty is achieved when the first ovulation ensues, and plasma progesterone levels are above 1 ng/ml. Indian buffalo generally reach puberty between the ages of 16 and 40 months, and on average, it takes over 2.5 years to reach sexual maturity. The Murrah breed of buffalo reaches puberty with an average age of 33 months; the Nilli Ravi buffalo breed reaches puberty at an average age of 32.5

months, and the Surti buffalo attains puberty at an average age of 45.5 months. The growth period of buffaloes after birth has a significant impact on their performance [2].

Cytokines play an important role in pubertal onset. Nutritional factors have an impact on pro-inflammatory and anti-inflammatory cytokines that are crucial for growth and reproduction. Complex inflammatory cytokine networks are involved in ovulation, embryo development, and implantation [3]. Since the beginning of phylogeny, Anti-Müllerian Hormone (AMH) has functioned as a modulator of germ cells and plays a critical role in inducing male sexual differentiation [4].

IFN- $\gamma$  plays a role in regulating immune cell recruitment, activation, and activity within the ovary, along with other modulations of the immune response [5]. IL-1 exerts significant regulatory control over gonadotropin activity in the reproductive system and influences granulosa cells. It promotes the proliferation and differentiation of granulosa cells, thus intricately modulating the dynamics of follicular development [6]. IL-6 plays a vital role in regulating cell proliferation, differentiation, follicle survival, atresia, and oocyte maturation in conjunction with other cytokines [7]. The presence of IL-13 in human ovarian tissues, particularly in granulosa cells (GCs) and theca cells (TCs), with the highest level detected during the luteal phase [8]. In rodent models, the effects of TGF- $\beta$  include stimulating the proliferation of granulosa cells, as well as

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promoting progesterone production and enhancing FSH-induced estradiol production [9]. During the early stages of follicle development, TNF- $\alpha$  reduces the ovary's sensitivity to gonadotropins, while in preovulatory follicles, TNF- $\alpha$  promotes steroidogenesis [10]. Anti-Müllerian hormone (AMH), also recognized as Müllerian inhibiting substance, belongs to the transforming growth factor-beta (TGF- $\beta$ ) family and is secreted from granulosa cells of developing follicles. Studies on AMH null mice indicate enhanced recruitment of primordial follicles into the growing pool, implying a suppressive impact of AMH on the transition from primordial to primary follicles [11].

Comparing cytokine and AMH levels during the first year of birth to predict puberty onset in buffaloes would strengthen the link between the observed changes and puberty timing. At the same time, focusing on cytokines with established links to reproductive maturity and puberty in buffaloes would be more informative. We hypothesize that changes in cytokine and AMH levels during prepuberty might provide valuable insights into the timing of puberty onset. This study aimed to investigate the changes in cytokine (IFN- $\gamma$ , IL-6, IL-1, IL-13, TNF- $\alpha$ , and TGF- $\beta$ ) and anti-Müllerian hormone (AMH) levels in prepubertal buffalo heifers (0-6 months) and compare them to pubertal heifers (1-year-old) to identify potential markers associated with puberty advancement.

The generated data will be analyzed to identify potential correlations between these levels and the timing of puberty onset. The study is planned to develop a non-invasive and reliable method for predicting puberty in buffalo heifers.

## MATERIALS AND METHODS

### Selection and Management of Animals

The study was conducted at the Directorate of Livestock Farms, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, from March to October 2023, and focused on buffalo calves and heifers. Buffalo calves and heifers were housed under optimal conditions, received proper nutrition, and were subject to comprehensive health management practices. The research was approved by the Institutional Animal Ethics Committee (IAEC) under letter no. GADVASU/2023/IAEC/69/11 dated 22/05/2023. Ten buffalo calves and ten buffalo heifers managed in a semi-intensive system with access to indoor and outdoor areas were incorporated into the

study. Buffalo calves, aged 0 to 6 months with an average weight of 35 kg, were fed a diet primarily consisting of milk, supplemented with either green fodder or silage based on availability, along with wheat straw, concentrates, and a mineral mixture. Prepubertal buffalo heifers, aged 12 months with an average weight of 200 kg, were primarily fed green fodder or silage (depending on availability), wheat straw, and concentrates, supplemented with the mineral mixture as per the ICAR (2013) feeding standards.

### Sampling

Jugular venous blood samples (5 ml) were collected from buffalo calves on the day of birth (day 0), day 15, day 30, day 60, day 90, day 120, day 150, and day 180. The buffalo heifers of one year of age were sampled for a single blood sample. The estimations were done for cytokines IFN- $\gamma$ , IL-6, IL-1, IL-13, TNF- $\alpha$ , and TGF- $\beta$ , and anti-Müllerian hormone AMH. The enzyme-linked immunosorbent assay (ELISA) kit was utilized for this cytokine and AMH estimation, procured from Bioassay Technology Laboratory, Shanghai Korain Biotech Co., Ltd, Shanghai, China.

### Statistical Analysis

Data generated from various ELISA estimations were presented as Mean  $\pm$  Standard Error of the Mean (SEM). Statistical analysis was conducted using SPSS software (22). Within-day comparisons were performed using repeated measures ANOVA, followed by pairwise comparisons using the least significant difference test. The significance level was set at  $P < 0.001$ . Karl Pearson's correlation coefficients were determined to establish the relationship between various cytokines and AMH.

## RESULTS

### Cytokines: IFN $\gamma$ , IL6, IL1, IL13, TNF $\alpha$ and TGF $\beta$ in Prepubertal Buffalo Calves and Heifers

Exploring the progression of immune and ovarian function in buffalo calves ( $n = 10$ , from birth to 180 days) and heifers ( $n = 10$ , aged 1 year) was done based on the assessment of the cytokines profiles viz. IFN- $\gamma$ , IL-6, IL-1, IL-13, TNF- $\alpha$ , and TGF- $\beta$ .

IFN- $\gamma$  concentrations exhibited a notable increase from birth ( $77.91 \pm 18.78$  ng/L) to day 150 of age ( $157.72 \pm 38.51$  ng/L), almost doubling their values. Throughout the 180-day period, the average IFN- $\gamma$  level stood at  $117.29 \pm 21.50$  ng/L, as detailed in Table

1. Specifically, serum IFN- $\gamma$  levels displayed a consistent rise from birth ( $77.91 \pm 18.78$  ng/L) to day 60 ( $127.31 \pm 23.42$  ng/L). However, there was a decline by day 90 ( $101.24 \pm 11.79$  ng/L), followed by another increase by day 150 ( $157.72 \pm 38.51$  ng/L). Notably, a sudden drop in IFN- $\gamma$  levels was observed on day 180 ( $94.62 \pm 19.33$  ng/L), as illustrated in Table 2 and ure 8. Furthermore, at 365 days (12 months) of age, serum IFN- $\gamma$  levels exhibited a slight elevation ( $114.17 \pm 25.28$  ng/L), as recorded in Table 2.

The concentration of IL-1 ranged from  $786.95 \pm 179.27$  ng/L on day 15 to  $1804.91 \pm 177.53$  ng/L on day 180, with an average over the 180-day period of  $1234.17 \pm 181.19$  ng/L. Notably, there was a decrease in levels on day 15, dropping from  $1234.22 \pm 226.75$  ng/L (on day 0) to  $786.95 \pm 179.27$  ng/L, followed by an increase on day 30 ( $1115.99 \pm 194.66$  ng/L), which remained relatively stable from days 30 to 120 ( $1150.13 \pm 125.08$  ng/L). Subsequently, elevated levels of IL-1 were observed on day 150 ( $1568.35 \pm 150.83$  ng/L), continuing to rise until day 180 ( $1804.91 \pm$

$177.53$  ng/L) (Table 1). These fluctuations in IL-1 levels were statistically significant ( $P < 0.01$ ) across all days. At 365 days (12 months), a slight reduction to  $1187.19 \pm 199.68$  ng/L was observed (Table 2).

IL-6 concentrations exhibited a range from  $84.31 \pm 38.79$  ng/L on day 90 to  $381.46 \pm 61.99$  ng/L on day 180, averaging  $183.90 \pm 37.64$  ng/L over the 180-day period (Table 1). From  $128.81 \pm 25.32$  ng/L at birth, IL-6 levels decreased to  $110.86 \pm 20.26$  ng/L by day 15. Subsequently, there was an increase to  $171.33 \pm 44.55$  ng/L by day 30, followed by a decline to  $84.31 \pm 38.79$  ng/L by day 60. Levels then rose to  $254.27 \pm 52.69$  ng/L on day 90, decreased to  $182.40 \pm 31.13$  ng/L by day 120, and further to  $157.82 \pm 26.43$  ng/L by day 150. Finally, IL-6 levels peaked at  $381.46 \pm 61.99$  ng/L on day 180 (Table 1). At 365 days (12 months) of age, the average IL-6 value was  $239.06 \pm 46.62$  ng/L (Table 2).

IL-13 levels varied between  $26.37 \pm 5.05$  ng/L on day 30 and  $57.76 \pm 19.13$  ng/L on day 15, averaging

**Table 1: Serum Cytokine Levels (Mean  $\pm$  SEM) Serum Cytokine Levels in Murrah Buffalo Calves (N=10) Up to 180 Days of Age**

Age of calf	Serum Cytokine						AMH
	IFN- $\gamma$	IL-1	IL-6	IL-13	TNF- $\alpha$	TGF- $\beta$	
Days	ng/L	ng/L	ng/L	ng/L	ng/mL	ng/mL	ng/L
0	77.91 $\pm$ 18.78	1234.22 <sup>bcd</sup> $\pm$ 226.75	128.81 <sup>bc</sup> $\pm$ 25.32	42.87 $\pm$ 7.01	9.65 <sup>bc</sup> $\pm$ 1.46	20.85 $\pm$ 3.70	19.95 $\pm$ 3.22
15	109.43 $\pm$ 21.93	786.95 <sup>d</sup> $\pm$ 179.27	110.86 <sup>c</sup> $\pm$ 20.26	57.76 $\pm$ 19.13	4.19 <sup>d</sup> $\pm$ 1.04	16.79 $\pm$ 4.05	33.49 $\pm$ 12.63
30	125.67 $\pm$ 22.76	1115.99 <sup>cd</sup> $\pm$ 194.66	171.33 <sup>b</sup> $\pm$ 44.55	26.37 $\pm$ 5.05	7.12 <sup>cd</sup> $\pm$ 1.71	18.37 $\pm$ 4.23	24.38 $\pm$ 5.12
60	127.31 $\pm$ 23.42	1007.43 <sup>bcd</sup> $\pm$ 218.13	84.31 <sup>c</sup> $\pm$ 38.79	39.10 $\pm$ 9.49	8.31 <sup>b</sup> $\pm$ 2.01	22.35 $\pm$ 4.57	22.34 $\pm$ 4.60
90	101.24 $\pm$ 11.79	1205.44 <sup>bcd</sup> $\pm$ 177.27	254.27 <sup>b</sup> $\pm$ 52.69	32.84 $\pm$ 7.65	7.55 <sup>c</sup> $\pm$ 1.48	21.48 $\pm$ 5.13	20.86 $\pm$ 3.47
120	144.44 $\pm$ 15.50	1150.13 <sup>b</sup> $\pm$ 125.08	182.40 <sup>bc</sup> $\pm$ 31.13	34.53 $\pm$ 4.93	9.71 <sup>bc</sup> $\pm$ 1.68	27.19 $\pm$ 4.97	21.31 $\pm$ 2.98
150	157.72 $\pm$ 38.51	1568.35 <sup>b</sup> $\pm$ 150.83	157.82 <sup>bc</sup> $\pm$ 26.43	31.56 $\pm$ 6.49	13.85 <sup>b</sup> $\pm$ 2.11	17.12 $\pm$ 4.07	14.42 $\pm$ 2.09
180	94.62 $\pm$ 19.33	1804.91 <sup>a</sup> $\pm$ 177.53	381.46 <sup>a</sup> $\pm$ 61.99	35.40 $\pm$ 6.32	19.85 <sup>a</sup> $\pm$ 1.97	15.37 $\pm$ 3.04	18.64 $\pm$ 3.99
<b>Average</b>	117.29 $\pm$ 21.50	1234.17 $\pm$ 181.19	183.90 $\pm$ 37.64	37.55 $\pm$ 8.25	10.02 $\pm$ 1.68	19.94 $\pm$ 3.88	4.60 $\pm$ 1.55
<b>P-value</b>	0.105 <sup>ns</sup>	0.001 <sup>**</sup>	0.001 <sup>**</sup>	0.212 <sup>ns</sup>	<0.001 <sup>**</sup>	0.249 <sup>ns</sup>	0.268 <sup>ns</sup>

ns- non-significant, \*\* P < 0.01 level, Means having different capital letters as superscripts differ significantly.

**Table 2: Serum Cytokine and AMH Levels (Mean  $\pm$  SEM) in Buffalo Heifers of 12 Months of Age**

		Parameter	Unit	Mean $\pm$ SEM
A.	Cytokine	1. Interferon- $\gamma$	ng/L	114.17 $\pm$ 25.28
		2. Interleukin-1	ng/L	1187.19 $\pm$ 199.68
		3. Interleukin-6	ng/L	239.06 $\pm$ 46.62
		4. Interleukin -13	ng/L	55.10 $\pm$ 18
		5. Tumor Necrosis Factor - $\alpha$	ng/mL	16.51 $\pm$ 3.16
		6. Transforming Growth Factor- $\beta$	ng/mL	9.07 $\pm$ 3.78
B.		Anti- Mullerian Hormone	ng/L	4.60 $\pm$ 1.55

37.55 ± 8.25 ng/L over the 180-day period. Serum IL-13 concentrations exhibited an increase from birth (42.87 ± 7.01 ng/L) to day 15 (57.76 ± 19.13 ng/L), followed by a decline to 26.37 ± 5.05 ng/L by day 30. Subsequently, levels rose again on day 60 (39.10 ± 9.49 ng/L) and remained consistent until day 180 (35.40 ± 6.32 ng/L) (Table 1). IL-13 levels significantly increased to 55.10 ± 18 ng/L by day 365 (12 months) of age (Table 2).

TNF-α levels ranged from 4.19 ± 1.04 ng/mL on day 15 to 19.85 ± 1.97 ng/mL by day 180 after birth, with an average of 10.02 ± 1.68 ng/mL over the 180-day period. There was a notable decrease in TNF-α levels from birth (9.65 ± 1.46 ng/mL) to day 15 (4.19 ± 1.04 ng/mL), followed by a subsequent increase until day 180 (19.85 ± 1.97 ng/mL). The differences in TNF-α values were statistically significant ( $P < 0.001$ ) (Table 1). The average TNF-α values obtained from birth to day 180 (16.51 ± 3.16 ng/mL) were comparable to those observed at 365 days (12 months) of age (Table 2).

TGF-β levels ranged from 15.37 ± 3.04 ng/mL on day 180 to 27.19 ± 4.97 ng/mL on day 120, with an average value of 19.94 ± 3.88 ng/mL over the 180-day period from birth. TGF-β exhibited a consistent decrease from the day of birth (20.85 ± 3.70 ng/mL) to day 15 (16.79 ± 4.05 ng/mL) of age. Subsequently, it steadily increased up to day 120 (27.19 ± 4.97 ng/mL) and then decreased to 15.37 ± 3.04 ng/mL by day 180 (Table 1). Furthermore, TGF-β levels drastically reduced to 9.07 ± 3.78 ng/mL at 12 months of age (Table 2).

### AMH in Prepubertal Buffalo Calves and Heifers

AMH concentrations varied from 14.42 ± 2.09 ng/L on day 150 to 33.49 ± 12.63 ng/L on day 15 of age in calves. The average AMH levels were 21.92 ± 4.76 ng/L over a period of 6 months. There was a steady

increase in AMH serum concentrations from the day of birth (19.95 ± 3.22 ng/L) to day 15 (33.49 ± 12.63 ng/L). However, these values significantly dropped to 14.42 ± 2.09 ng/L on day 150 and then increased slightly to 18.64 ± 3.99 ng/L on day 180 (Table 1). Furthermore, serum levels of AMH experienced a drastic reduction at 12 months of age (4.60 ± 1.55 ng/L) (Table 2).

### Karl Pearson Correlation Coefficient for Cytokines and AMH in Buffalo Calves and Heifers

Karl Pearson correlations were observed among different cytokines and AMH. AMH exhibited a positive correlation with IL-13 ( $r = 0.266$ ,  $P < 0.05$ ) and TGF-β ( $r = 0.270$ ,  $P < 0.05$ ), while it was highly negatively correlated with IFN-γ ( $r = -0.219$ ,  $P < 0.05$ ) and TNF-α ( $r = -0.209$ ,  $P < 0.05$ ). Additionally, AMH showed negative correlations with IL-1 ( $r = -0.011$ ) and IL-6 ( $r = -0.041$ ). However, except for AMH, the correlations with other cytokines were not statistically significant. IFN-γ exhibited positive correlations with IL-13 ( $r = 0.089$ ), TGF-β ( $r = 0.067$ ), and TNF-α ( $r = 0.102$ ), while it showed negative correlations with IL-1 ( $r = -0.122$ ) and IL-6 ( $r = -0.150$ ). IL-1, besides the aforementioned correlations, also showed positive correlations with IL-6 ( $r = 0.334$ ,  $P < 0.01$ ), IL-13 ( $r = 0.021$ ), and TNF-α ( $r = 0.404$ ,  $P < 0.01$ ), and a negative correlation with TGF-β ( $r = -0.220$ ,  $P < 0.05$ ). IL-6 exhibited negative correlations with IL-3 ( $r = -0.012$ ) and TGF-β ( $r = -0.187$ ), while it showed a positive correlation with TNF-α in addition to the above. IL-13 also displayed positive correlations with TGF-β ( $r = 0.123$ ) and TNF-α ( $r = 0.109$ ), whereas TGF-β showed a highly significant negative correlation with TNF-α ( $r = -0.328$ ,  $P < 0.01$ ). These correlations are depicted in Table 3.

### DISCUSSION

Macrophages and lymphocytes within the ovarian immune system serve as signaling molecules,

**Table 3: Karl Pearson Correlation Coefficient of Cytokines and Amhin Buffalo Calves (N=10) and Heifers (N=10) Up To 1 Year of Age**

Variable	AMH	IFN-γ	IL-1	IL-6	IL-13	TGF-β	TNF-α
AMH	1	-0.219*	-0.011	-0.041	0.266*	0.270*	-0.209*
IFN-γ	-0.219*	1	-0.122	-0.150	0.089	0.067	0.102
IL-1	-0.011	-0.122	1	0.334**	0.021	-0.220*	0.404**
IL-6	-0.041	-0.150	0.334**	1	-0.012	-0.187	0.326**
IL-13	0.266*	0.089	0.021	-0.012	1	0.123	0.109
TGF-β	0.270*	0.067	-0.220*	-0.187	0.123	1	-0.328**
TNF-α	-0.209*	0.102	0.404**	0.326**	0.109	-0.328**	1

\*\*  $P < 0.01$  level; \*  $P < 0.05$  level

releasing cytokines such as IFN- $\gamma$ , TNF- $\alpha$ , IL-1, and IL-6, among others. These cytokines play a crucial role in ovarian functions, impacting processes like egg development, ovulation, and progesterone hormone production [12]. Within the ovarian follicle, these cytokines are locally produced and diffuse to establish chemotactic gradients in a paracrine/autocrine manner. They act at low concentrations and have a short half-life that does not significantly affect systemic profiles.

The research on the role of macrophages and lymphocytes in the ovarian immune system holds great promise for understanding ovarian function and potential dysfunction. Some limitations and potential biases that may influence the results are Limited *In vivo* Studies, Cytokine Measurement Challenges as cytokines are short-lived and act locally within the ovary, Individual Variability regarding ovarian function and immune response, Specificity of Cytokine Action having pleiotropic effects depending on the cell type and context.

Addressing these limitations is crucial for Refining our understanding of the precise roles of macrophages, lymphocytes, and cytokines in ovarian function, developing more accurate methods to measure and analyze cytokine activity within the ovary, and designing future studies that account for individual variability and considering the broader immune system context.

Our study estimates IFN- $\gamma$  levels in buffalo calves for the first time up to 1 year of age. Compared to IFN levels reported by Hisaeda and co-workers [13] in cows with coliform mastitis, our study reports much lower levels and also reported undetectable levels in healthy cattle, with values ranging from 50.7 $\pm$ 14.7 ng/mL to 39.8 $\pm$ 9.7 ng/mL in mastitic animals. The lower IFN- $\gamma$  levels observed in our study may be due to neutrophils being the primary defense cells, with IFN- $\gamma$  acting as an activator for neutrophils. Activation of Interferon- $\gamma$  enhances their ability to engulf and eliminate pathogens such as viruses, bacteria, and parasites.

Concentrations of IFN- $\gamma$  in plasma were significantly lower in newborns (0.42 pg/mL) compared to children (1.2 pg/mL) from healthy non-atopic parents, regardless of breastfeeding or formula feeding [14]. This difference may be attributed to type 2 immunity prevailing at birth, while type 1 immunity dominates during childhood. Type 2 immunity persists throughout the neonatal period, irrespective of feeding mode, promoting the development of adaptive immunity,

where B and T cells learn to target and remember specific threats. In our study, IFN concentration fluctuated on subsequent days throughout the year, likely influenced by nutritional and health management practices, including vaccination.

IL-1 levels in buffalo calves and heifers have not been previously documented in the literature. Existing references provide reference values for other species and conditions. The IL-1 concentrations observed in our study were notably higher compared to findings by El-Bahr and El-Deeb [15], who reported IL-1 levels in healthy and broncho-pneumonic buffalo calves aged 9 months as 102.43 $\pm$ 2.45 pg/mL and 640.43 $\pm$ 20 pg/mL, respectively. In calves aged 0-6 months with various conditions such as control, pneumonic, pneumo-enteritis, and enteritis, IL-1 concentrations were reported as 14.17 $\pm$ 1.04, 40.83 $\pm$ 3.75, 57.26 $\pm$ 4.40, and 55.60 $\pm$ 7.91 pg/mL, respectively, which were lower than the IL-1 concentrations observed in our study.

IL-1 serves as the primary mediator of the body's early inflammatory response to infection, attracting immune cells to the site of infection, priming them to combat the virus, and enhancing the generation of additional immune molecules. Additionally, it aids in activating B and T cells, which is crucial for producing specific antibodies and establishing immunological memory against the pathogen [16]. For instance, in Anatolian buffaloes aged 11 months infected with dermatophytosis compared to healthy controls, IL-1 levels were reported as 186.22  $\pm$  0.22 pg/mL and 74.04  $\pm$  0.90 pg/mL, respectively. The higher IL-1 concentration in the dermatophyte-affected population indicates inflammation due to dermatophytosis [17].

Notably, studies investigating the effect of IL-1 from birth to 1 year of age in prepubertal calves and heifers are lacking in the literature. The higher IL-1 values observed in our study, with significant differences across the days, may be attributed to the health status and vaccination schedule implemented at the farm.

IL-6 levels in buffalo calves and heifers have not been documented in existing literature. In the study, IL-6 levels observed throughout the study period were higher compared to those reported by Kabu and Sayin [17], who found IL-6 levels of 32.45  $\pm$  0.20 pg/mL and 55.94  $\pm$  0.50 pg/mL in control and dermatophyte-affected 11-month-old Anatolian buffaloes, respectively. Elevated IL-6 levels in infected animals can be attributed to their immune response.

Furthermore, IL-6 levels were found to be greater in pregnant cows ( $515.5 \pm 75.2$  pg/mL) compared to non-pregnant ones ( $375.3 \pm 262.9$  pg/mL) in uterine flushes and  $403.1 \pm 180.4$  pg/mL and  $289.6 \pm 109.7$  pg/mL, respectively, in vaginal flushes [18]. IL-6 acts as a pro-inflammatory cytokine regulating various inflammatory processes and acute phase responses [19, 20]. It has also been classified as an embryokine, with the oviduct and endometrium utilizing these embryokines to control the development of bovine preimplantation embryos [21-23].

In studies on buffalo and cattle infested with Fasciolosis, IL-6 levels were found to increase [24], and IL-6 is associated with Antibody-Dependent Cell-mediated Cytotoxicity (ADCC) against neutrophils [25]. For instance, IL-6 levels in calves affected with enteritis, pneumo-enteritis, pneumonia, and the control groups were reported as  $22.14 \pm 2.82$  pg/mL,  $16.40 \pm 0.90$  pg/mL,  $12.01 \pm 1.18$  pg/mL, and  $5.44 \pm 0.82$  pg/mL, respectively [16]. The discrepancy between our study's IL-6 values in calves and heifers and those reported in previous studies could be due to differences in the health status and vaccination schedules followed on the farm.

There is a lack of existing literature reporting the estimation of IL-13 levels in buffalo calves and heifers, making this study the first of its kind. Unlike in human children, where no age-related effects on IL-13 production have been documented [26], these findings suggest a reduction in IL-13 production with advancing age. IL-13 functions as a mediator of allergic asthma, regulating mucus secretion, airway hyperresponsiveness, and eosinophilic inflammation [27].

TNF- $\alpha$  levels in buffalo calves and heifers have not been extensively documented in published literature. The values obtained in our study were notably higher compared to those reported by Kabu and co-workers [16] for calves infected with pneumo-enteritis, enteritis, pneumonia, and control groups, which were  $0.28 \pm 0.02$  ng/mL,  $0.29 \pm 0.05$  ng/mL,  $0.31 \pm 0.03$  ng/mL, and  $0.10 \pm 0.01$  ng/mL, respectively. The higher values observed in our study can be attributed to the inflammatory properties of this cytokine. It is likely that the reported values in our study are influenced by the health and vaccination status of the calves on the farm.

There are no existing reports on cytokine estimation from birth to 1 year of age in buffalo heifers. While some studies have investigated cytokine levels in

pregnant cattle, such as TGF- $\beta$  concentrations being higher in the pregnant uterus ( $44 \pm 13.4$  pg/mL) compared to the non-pregnant uterus ( $14.7 \pm 4.9$  pg/mL) [18], these findings are higher compared to our study's results for 12 months of age. The differences observed in our study may be associated with the health and immunization status of the farm's calves and heifers.

AMH levels have been studied in cattle, but there is currently no published literature on this topic for buffaloes. However, the values observed in our study were notably lower compared to those reported by Rota and co-workers [28], who found plasma AMH values in female bovines aged 1 year to be up to 90 ng/mL. Additionally, Batista *et al.* [29] noted that plasma AMH levels are lower in cycling heifers than in non-cycling ones, and AMH levels were higher in calves than in heifers. Furthermore, AMH concentrations have been shown to accurately predict the amount of ovarian follicle reserve in heifers and cows [30].

The observed correlations between anti-Müllerian hormone (AMH) and various cytokines likely stem from the intricate relationship between the immune system and reproductive physiology.

There is a positive association between AMH, TGF- $\beta$  and IL-13. TGF- $\beta$  and IL-13 are renowned for their roles in immunomodulation and tissue healing. TGF- $\beta$ , crucial for ovarian follicle development and AMH production, plays a vital role [31]. IL-13 has been implicated in ovarian function and aids in regulating AMH levels [32].

Conversely, AMH exhibits a negative correlation with TNF- $\alpha$  and IFN- $\gamma$ . Research has shown that pro-inflammatory cytokines like TNF- $\alpha$  and IFN- $\gamma$  suppress AMH production. TNF- $\alpha$  inhibits AMH synthesis in granulosa cells, while IFN- $\gamma$  prevents human granulosa cells from expressing AMH [33].

IL-1 displays a positive correlation with TNF- $\alpha$  and IL-6. IL-1 is potent in triggering inflammatory reactions and boosting TNF- $\alpha$  and IL-6 production [34]. These cytokines often collaborate to amplify inflammatory signals, contributing to the development of various inflammatory disorders. Notably, IL-1's negative relation with TGF- $\beta$  in this study suggests a regulatory connection, where IL-1 downregulates TGF- $\beta$  production and function [35].

IL-6 exhibits positive correlations with TNF- $\alpha$  and IL-1, as both TNF- $\alpha$  and IL-1 stimulate IL-6 synthesis [36].

The positive associations among cytokines underscore their interdependence in inflammatory reactions. The positive correlation between AMH and TGF- $\beta$  suggests a favorable relationship, indicating a potential role for TGF- $\beta$  in regulating AMH synthesis, given its association with ovarian function and follicular development [37]. Conversely, TGF- $\beta$  demonstrates a negative connection with TNF- $\alpha$  and IL-1, as it inhibits the synthesis and function of pro-inflammatory cytokines [38]. This antagonistic relationship highlights the interplay between TGF- $\beta$ -mediated immunosuppression and the pro-inflammatory effects of IL-1 and TNF- $\alpha$ .

## CONCLUSIONS

Upon birth, cytokine levels in serum indicated a mixed pattern, with lower levels observed for IFN- $\gamma$ , IL-6, and IL-13, whereas IL-1, TNF- $\alpha$ , and TGF- $\beta$  exhibited higher concentrations. Throughout the study period, levels of IFN- $\gamma$ , IL-13, and TGF- $\beta$  remained relatively consistent, whereas IL-1, IL-6, and TNF- $\alpha$  exhibited an increasing trend, particularly evident by day 180 of the buffalo calf's age. Higher levels ( $P < 0.01$ ) of IL-1, TNF- $\alpha$ , and IL-6 were observed from birth up to 180 days of age, as well as at day 365 of age. Anti-Müllerian hormone (AMH) levels were almost similar from birth to day 180, with a notable increase immediately after birth (15 days of age), with levels reaching  $33.49 \pm 12.63$  ng/L. At the end of the first year, AMH levels reduced considerably to  $4.60 \pm 1.55$  ng/L. Implications of the Karl Pearson correlation coefficient reveal that the AMH levels are negatively correlated with IFN- $\gamma$  and TNF- $\alpha$ . In contrast, it shows a positive correlation with IL-13 and TGF- $\beta$ . Therefore, IFN- $\gamma$  and TNF- $\alpha$  levels are indicators for the reduction in AMH levels. Hence, the setting up of puberty can be taken as a predictive marker for ensuing puberty in buffalo heifers.

## RECOMMENDATIONS

Specific recommendations based on the study's findings: 1) Investigate the Functional Significance of Cytokines in buffalo puberty onset and whether these cytokines directly influence the decline of AMH or are they markers of a broader physiological process related to puberty. 2) Explore Mechanisms of Action, as the study suggests a negative correlation between IFN- $\gamma$ /TNF- $\alpha$  and AMH and a positive correlation between IL-13/TGF- $\beta$  and AMH. Further research should explore the mechanisms by which these cytokines might influence AMH levels; 3) Validate Findings in a

Larger Sample Size, as the current study used a relatively small sample size (10 calves and 10 heifers), to strengthen the generalizability of the findings; 4) Investigate Cytokine Profiles in Different Breeds, to investigate cytokine profiles in different buffalo breeds to assess potential variations; 5) Longitudinal Hormone Monitoring, throughout the prepubertal and pubertal stages might provide a more complete picture of hormonal changes; 6) Investigate the Impact of Environmental Factors, like nutrition or stress on cytokine profiles and puberty timing could be a valuable next step.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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