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Seasonal Comparative Analysis of Follicular Interleukin Concentration and Expression of IL-1RII, IL-4 and IL-1RA in Cystic Follicles Versus Normal Preovulatory Follicles in Buffalo

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ABSTRACT

Background: Cystic ovarian disease (COD) is a prevalent cause of infertility in buffalo, necessitating a deeper understanding of the underlying molecular mechanisms. This study aimed to investigate the expression levels of IL-1RII, IL-1RA and IL-4 cytokines in naturally occurring ovarian follicular cysts obtained from a slaughter house.

Methods: The relative gene expression was assessed using real-time PCR (RT-PCR) while concentrations of IL-1 and IL-4 were quantified in follicular fluid *via* ELISA.

Result: The results revealed that the IL-4 gene was expressed in both normal and cystic follicles, with slightly higher expression observed in cystic follicles, albeit without statistical significance. Interestingly, higher concentrations of IL-1 and IL-4 were found in normal follicles compared to cystic follicles, regardless of the season, with no significant variations between seasons within the same follicle type. These findings provide valuable insights into the altered cytokine expression and hormonal profiles associated with cystic ovarian disease in buffalo. Further research is warranted to elucidate the precise roles of these cytokines and hormones in the pathophysiology of COD, potentially leading to improved diagnostic and therapeutic strategies for this infertility disorder in buffalo.

Key words: Buffalo, Cystic ovarian disease, Follicular fluid, Gene expression, Infertility, Interleukins.

INTRODUCTION

Buffaloes hold the distinction of being the second-largest milk producer worldwide and contribute to over one-third of Asia's total milk production (Bandyopadhyay *et al*., 2003). Unfortunately, infertility, low fertility and sterility affect a significant proportion of cattle and buffaloes in India, leading to the slaughter of 18 to 40 percent of these animals annually and directly impacting the genetic resources of farmers (Sharma *et al*., 1993). The reproductive capacity of buffaloes can be significantly impacted by different types of ovarian cysts, including follicular cysts, luteal cysts and cystic corpora lutea. These conditions collectively form what is known as cystic ovarian disease. Among the causes of subfertility in dairy cattle, cystic ovarian disease (COD) stands out as a significant factor (Garverick, 1997). The presence of cystic ovarian follicles remains a significant cause of reproductive failure in lactating dairy cows and can also lead to reduced milk supply and production potential (Das and Khan, 2010).

The etiopathogenesis of ovarian cysts in dairy cattle is a complex process involving changes in multiple physiological processes such as folliculogenesis, steroidogenesis and ovulation. Additionally, factors like stress, herd management, nutritional status, body condition, metabolic disorders, altered mRNA expression and Matrix Metalloproteinases and their inhibitors contribute to cyst development (Yeo and Colledge, 2018). Cytokines play a crucial role in the formation of ovarian follicles by regulating the proliferation, differentiation and degeneration of various ovarian cellular components (Nash *et al*., 1999). They have also been shown to affect various physiological processes, including ovulation and are expressed during immunopathological responses (Dhillo *et al*., 2006).

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Despite some studies addressing cystic follicles in cattle, no research has been conducted on this disorder in buffaloes, even though the incidence is comparable. Moreover, studies related to interleukins and their role in the development of cystic ovarian degeneration (COD) in cattle have primarily focused on induced cystic ovaries, rather than *in vivo* observations. The dairy sector faces substantial financial setbacks due to the extended periods between calving and conception, as well as the lengthened intervals between consecutive calvings, both of which result from this disease. These delays not only reduce the productivity and efficiency of dairy operations but also often lead to the necessity of culling affected animals from the herd. This removal of diseased animals further compounds the economic impact, as it reduces the overall number of productive cows, leading to decreased milk production and increased costs for herd replacement and management. (Peter, 2004; Cattaneo *et al*., 2014).

The incidence of cystic ovarian disease in dairy cattle ranges from 5 to 30%, while in buffaloes, it varies from 2.7 to 30% (Ahmad and Khan, 1993; Hatipoglu *et al*., 2000; Feyissa, 2004; Azawi, 2009). Cystic ovarian disease is characterized by the presence of one or more follicles exceeding 20 mm in diameter in the ovaries, persisting for up to 10 days without luteal tissue, thereby interrupting the normal reproductive cycle (Silvia *et al*., 2002). Although the pathogenesis and mechanisms of cyst formation are not fully understood, it is widely accepted that altered function of the hypothalamic-pituitary-ovarian axis is a significant component in the etiopathogenesis of COD (Silvia *et al*., 2002). However, the persistence of follicles over time is associated with intra-ovarian factors.

Numerous studies have investigated the presence of different cytokines in normal ovaries. However, the alteration of these cytokines during ovarian dysfunction remains largely unexplored. Considering the multitude of factors involved in ovulation, it is hypothesized that the alteration of one or more components in this process may contribute to the pathogenesis of COD (Baravalle *et al*., 2015). Given the buffalo's significant contribution to milk production in India, particularly in regions such as Haryana, there is an urgent need to identify immunological markers in buffaloes with cystic ovaries. This is driven by the increasing prevalence of cystic ovarian degeneration in buffaloes and the limited knowledge surrounding the immunology of cystic ovaries in this species. Furthermore, since the ovary is a site of inflammatory reactions and ovarian cells can be potent sources and targets of cytokines, this study aimed to examine the expression of IL-1RII, IL-4 and IL-1RA in ovarian cells obtained from preovulatory and cystic follicles in buffaloes. considering the buffalo's breeding tendencies during winter and reduced oestrus manifestation resulting in decreased pregnancy rates during summer, this study also aimed to explore seasonal variations in interleukin expression and their follicular fluid concentration.

MATERIALS AND METHODS

This study was carried out in the Department of Animal Biotechnology, College of Veterinary Sciences, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar. The study was conducted following all the procedures approved by university IAEC.

Collection of ovaries

A total of 1800 buffalo ovaries were collected from the Municipal Corporation of Delhi Slaughter House in both the summer (May-June) and winter (December-January) seasons. The ovaries were transported to the laboratory within 4-5 hours in a thermos flask containing sterilized normal saline solution (NSS) supplemented with antibiotics.

Collection of follicular fluid and follicular wall

Pre-ovulatory follicles (12-14 mm in diameter) and cystic follicles (≥20 mm in diameter) were included in the study. The follicles were categorized based on their size and the follicular fluid was individually aspirated using a disposable syringe attached to an 18-gauge needle. The follicular wall was excised and the follicular samples were grouped into categories based on size and season.

RNA extraction and quantification

Total RNA was isolated from the follicular wall samples using an RNeasy kit. The concentration and quality of the extracted RNA were assessed.

Primer designing for RT-qpcr experiments

The primers for IL-1RII, IL-4, IL-1RA and GAPDH were designed after multiple sequence alignment of sequences available in NCBI database using online available Primer 3 software. The list of primers used in the experiment is given in Table 1.

Relative quantification by Livak's Method (ΔΔCq Method)

For the analysis of expression profile of different genes, real-time PCR was carried out using StepOneplus qPCR system (Applied biosystem). For the real-time PCR reaction, Luna Universal One-Step SYBR Green RT-qPCR kit (LUNA) was used and all the instructions were followed as per the manufacturer's instructions. The reaction for the target gene (IL-1RII, IL-4, IL-1ra) and the endogenous control (GAPDH) gene was carried out in triplicates along with non-template control as a negative control for each sample. Data were analyzed via SDS version 2 software, applying Livak's method (Livak and Schmittgen, 2001) to calculate fold changes.

Analysis of follicular fluid for cytokine

The concentrations of interleukin 1 (IL-1) and interleukin 4 (IL-4) in the follicular fluid were determined using commercially available bvine ELISA kits.

Statistical analysis

Statistical analyses was performed using SPSS version 23. RT-qPCR data were presented as fold change (RQ),

with significance defined as a fold change exceeding two according to Applied Biosystem (2008). Hormonal concentrations were reported as mean±SE.

A repeated measures ANOVA assessed variation among ovulatory follicle categories within seasons, with Duncan's post-hoc test. ANOVA compared differences between summer and winter seasons.

RESULTS AND DISCUSSION

Analysis of expression of *IL1RII, IL4* and *IL1RA* genes

The extracted RNA underwent quantitative and qualitative analysis using a Smartspec spectrophotometer (BioRad). To minimize variation within the groups, the RNA from all five groups (each containing 6 follicles) was pooled. The level of target mRNA was determined using the comparative -HCq method. For relative quantification, RT-qPCR was performed in triplicates for each group. Self-designed primers specific to the genes of interest (IL-1RII, IL-4 and IL-1RA) and GAPDH as the endogenous control were used. The preparation of mastermix (Table 2) and the cycling conditions (Table 3) for one step RT-qPCR was normalized separately for each gene along-with GAPDH gene for the best performance in terms of sensitivity and reproducibility to get best amplification of the gene of interest.

Expression profiling of IL-1RII, IL-4 and IL-1RA revealed no significant change between normal and cystic follicles in both seasons. However, there was a downregulation of gene expression in cystic follicles. In the summer season, the expression level of IL1RII did not show a specific pattern and was similar to that of the winter season in both normal and cystic follicles. The expression levels of IL-1RII were also similar between normal pre-ovulatory follicles and cystic follicles (Fig 1). In the winter season, the expression level of IL-1RII was 1.37 in normal pre-ovulatory follicles compared to 1.035 in cystic follicles, but the difference was not significant.

The expression level of IL-4 did not significantly differ between normal and cystic follicles in both summer and winter seasons. There was a slight upregulation of IL-4 expression in cystic follicles compared to normal pre-ovulatory sized follicles in both seasons, but the difference was not statistically significant (Fig 2).

Similarly, the expression level of IL1RA showed no significant difference between normal pre-ovulatory sized follicles and cystic follicles during summer and winter seasons. However, in winter, there was a marginal upregulation of IL-1RA expression in normal pre-ovulatory follicles compared to cystic follicles (Fig 3). Nevertheless, the expression of IL-1RA was similar between winter and summer seasons for the same category of follicles.

Seasonal variation in IL1 and IL4 concentrations in follicular fluid (FF)

The follicular fluid IL1 concentration showed no significant difference ($p>0.05$) between winter and summer seasons in both normal and cystic follicles. However, the IL-1 concentration was significantly higher ($p<0.05$) in cystic follicles (193.47±4.43 and 197.60±6.22 ng/L) compared to normal follicles (148.74±8.98 and 141.97±8.65 ng/L) in winter and summer seasons, respectively (Fig 4).

Similarly, the follicular fluid IL-4 concentration was higher ($p<0.05$) in cystic follicles (193±7.42 and 186.44± 6.71 ng/L) compared to normal follicles (124.59±10.64 and 142.43±7.65 ng/L) in winter and summer seasons, respectively (Fig 5). However, there was no significant difference ($p>0.05$) in intrafollicular fluid concentrations of IL-4 between summer and winter seasons for both normal and cystic follicles.

Table 1: Primer designing for one step RT-qPCR experiments.

Gene	Primers	Sequence (5′-3′)	Product size (bp)
IL-1RII	F	GGTCAACAACACCCTGGTGGAC	177
	R	GCAGCAGACACTCCAGCATGAC	
IL-4	F	AACCTTCTGCAGGGTTGGAATTG	141
	R	CTGCTCGTCTTGGCTTCATTC	
IL-1RA	F	GGTACCCATCGAACCCCATAC	118
	R	GTCAGTGATGTTCACGGCCTC	
GAPDH	F	AGATGGTGAAGGTCGGAGTG	136
	R	GATCTCGCTCCTGGAAGATG	

Table 2: Preparation of mastermix for one step RT-qPCR.

Mastermix	IL-IRII (µL)	IL-4 (µL)	IL-IRA (µL)	GAPDH (µL)
2X Reaction mix (LUNA Universal one step SYBR)	5	5	5	5
Sense primer (10 µM)	0.25	0.25	0.25	0.25
Anti-sense primer (10 µM)	0.25	0.25	0.25	0.25
RT Enzyme mix (20 X)	0.5	0.5	0.5	0.5
Nuclease free water	2.5	2.5	2.5	2.5
Template	1.5	1.5	1.5	1.5

The etiopathogenesis of ovarian cysts in dairy cattle involves complex changes in physiological processes such as folliculogenesis, steroidogenesis and ovulation. Contributing factors include stress, herd management, nutrition, metabolic disorders, altered mRNA expression and the roles of Matrix Metalloproteinases and their inhibitors. However, the exact cause of ovarian cysts remains challenging to pinpoint due to the involvement of multiple interrelated pathways (Yeo and Colledge, 2018).

So, the present study investigated, for the first time Interleukin expression in normal preovulatory and cystic ovarian follicles of buffalo in summer and winter season. Though there are few studies related to cystic follicles in cattle but in buffalo no such study has been carried out and due to the incidence of this disorder as par in cattle this need to be investigated. The studies related to interleukins and their role in development of COD in cattle are on induced cystic ovaries and not *in vivo*. This study in buffalo is in naturally occurring cystic follicles collected from slaughtered animals. As water buffalo is a seasonal breeder so seasonal variation of this disorder also remains an important topic to be investigated. So, this study was designed to know about the role of interleukins in etiopathology of cystic follicles in ovarian follicles of buffalo and how they variate between summer (long days) and winter (short days) seasons.

Numerous studies have demonstrated that cytokines affect various physiological processes, including ovulation, in addition to being expressed during immunopathological responses (Dhillo *et al*., 2006). This is because cytokines control the formation of ovarian follicles by controlling the proliferation, differentiation and degeneration of many ovarian cellular components (Nash *et al*., 1999). In this study, the expression levels of IL-1RII, IL-4 and IL-1RA were examined in the follicular walls of normal preovulatory-sized and cystic follicles. No significant differences were found in the expression of these genes between the two types of follicles. However, there was a slightly higher expression of IL-1RII ligand in normal follicles compared to cystic follicles during winter, although without statistical significance. IL-1RII acts as a decoy receptor, modulating the biological activity of IL-1 by binding less efficiently to IL-1RA and serving as a molecular trap for IL-1α and IL-1β. IL-1RII expression was found to be higher in atretic and cystic follicles compared to other follicular categories examined in cattle (Stassi *et al*., 2018).

IL-4 gene expression was observed in both normal and cystic follicles during both seasons, with slightly higher expression in summer cystic follicles compared to normal preovulatory-sized follicles. IL-4 is an anti-inflammatory cytokine that can interact with IL-4 receptors to reduce inflammation (Eyestone *et al*., 1984; Lopez-Díaz *et al*., 1992; Garverick, 1997; Ribadu *et al*., 2000; Espey *et al*., 2004). Increased IL-4 expression in cystic follicles may indicate its role in blocking the ovulatory inflammatory process and preventing ovulation.

Table 3: Cycling conditions for one step RT-qPCR.

Genes	Holding	Cycle (45)			Melt curve
ILIRII	55°C for 30 minutes	95°C for 1 minute	95°C for 10 seconds	60°C for 60 seconds	60-95°C for 30 seconds	As per instrument instructions
IL4	55°C for 30 minutes	95°C for 1 minute	95°C for 10 seconds	60°C for 60 seconds	60-95°C for 30 seconds	As per instrument instructions
ILIRA	55°C for 30 minutes	95°C for 1 minute	95°C for 10 seconds	60°C for 60 seconds	60-95°C for 30 seconds	As per instrument instructions
GAPDH	55°C for 30 minutes	95°C for 1 minute	95°C for 10 seconds	60°C for 60 seconds	60-95°C for 30 seconds	As per instrument instructions

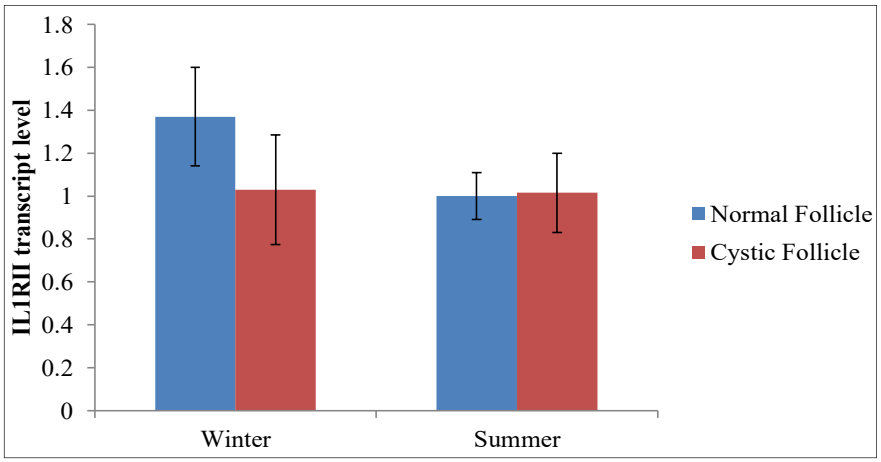

Fig 1: Relative mRNA transcript levels (mean±SE) of IL-1RII gene in normal pre-ovulatory follicles and cystic follicles in winter and summer season.

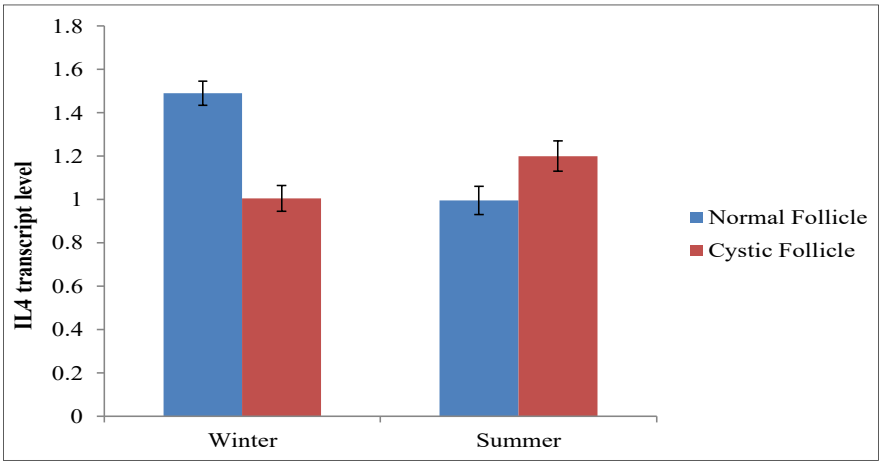

Fig 2: Relative mRNA transcript expression levels (mean±SE) of IL-4 gene in normal preovulatory sized follicles and cystic follicles during summer and winter season.

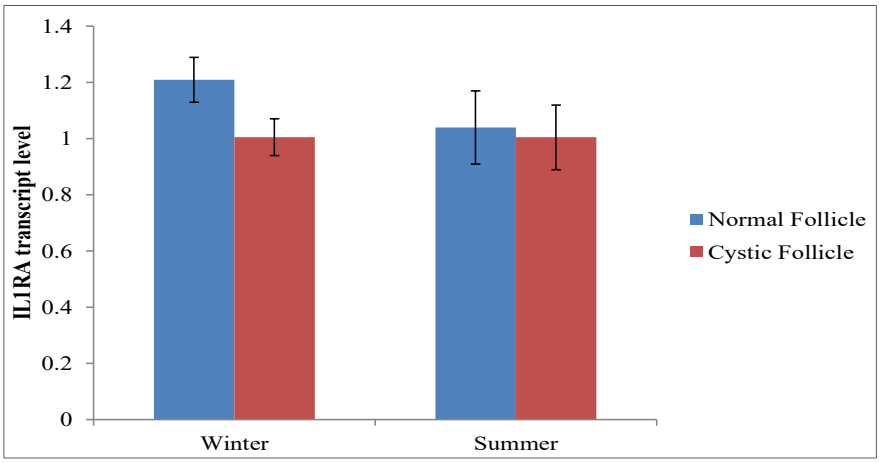

Fig 3: Relative expression of mRNA transcript (mean±SE) of IL-1RA gene in normal and cystic follicles during summer and winter season.

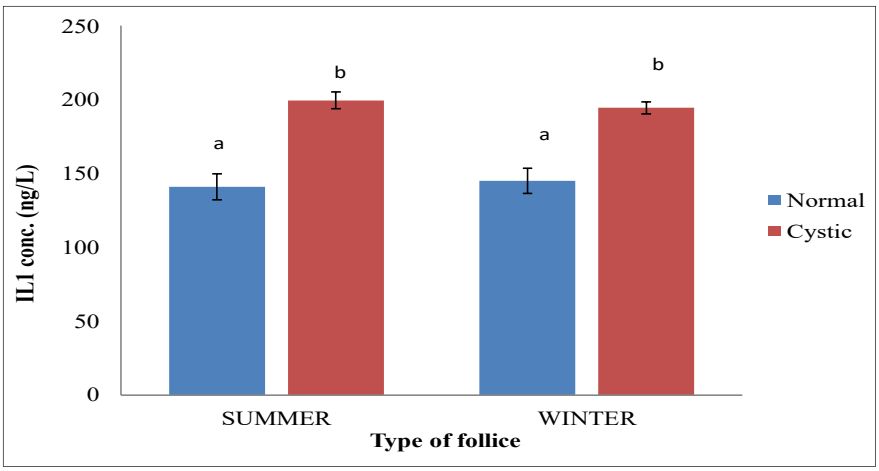

Fig 4: The intrafollicular concentration of IL-1 (ng/L) in normal and cystic follicles during the summer and winter seasons shows significant differences[a,b] ($p<0.05$) between normal and cystic follicles within the same season.

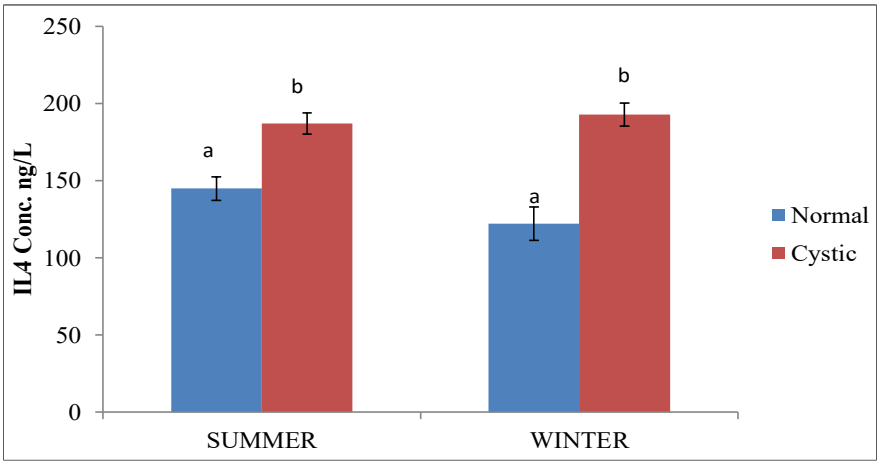

Fig 5: The intrafollicular concentration of IL-4 (ng/L) in normal and cystic follicles during the summer and winter seasons shows significant differences[a,b] ($p<0.05$) between normal and cystic follicles within the same season.

IL1RA, an antagonist of IL-1 ligands, was expressed in both types of follicles without significant differences between them or between seasons. IL-1RA binds to IL-1RI and suppresses IL-1 signaling, reducing the effects of IL-1. IL-1RA expression is involved in the anti-inflammatory action of regulators such as cytokines, glucocorticoids and prostaglandins (Passos *et al*., 2016).

The study also investigated the intra-follicular concentration of IL-4 and IL-1 in normal preovulatory-sized follicles and cystic follicles. Intra-follicular IL-4 concentrations was higher in cystic follicles than in normal follicles during both seasons. Similarly, IL-1 concentration was higher in cystic follicles than in normal follicles, indicating their association with ovarian physiology and potential role in the ovulation process. The findings suggest the involvement of interleukins in the development of cystic follicles and their potential role in modulating the ovulatory process. However, further research is needed to elucidate the exact mechanisms and interactions between interleukins, hormones and other factors in the pathogenesis of ovarian cysts in buffalo.

Conclusion

In this study, interleukin expression in normal preovulatory-sized and cystic follicles of buffalo was investigated. Although no significant differences were found in the expression levels of interleukins between normal and cystic follicles, higher intra-follicular concentrations of IL-4 and IL-1 were observed in cystic follicles. These findings provide insights into the potential role of interleukins and hormonal factors in the development of cystic follicles in buffalo. Further research is required to elucidate the underlying mechanisms and interactions involved in this process.

Conflict of interest

The authors have no conflict of interest to declare.

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