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Expression of Chitinase-like 3 Protein 1 (CHI3L1) in Buffalo Cumulus-oocyte Complexes during *in vitro* Maturation

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ABSTRACT

Background: Buffaloes (*Bubalus bubalis*) are a vital livestock species in South Asia, significantly contributing to milk production and agricultural sustainability. However, reproductive inefficiency remains a significant challenge, adversely impacting productivity. In vitro maturation (IVM) of oocytes is a critical technique for improving reproductive efficiency in buffaloes. Chitinase-3-like Protein 1 (CHI3L1), known for its roles in tissue remodelling and inflammation, has emerged as a potential regulator in reproductive processes. This study investigates the expression of CHI3L1 in buffalo cumulus-oocyte complexes (COCs) during IVM to explore its role in oocyte maturation.

Methods: Buffalo ovaries were collected post-slaughter and COCs were retrieved and cultured in TCM 199 medium supplemented with FSH, LH and estradiol. The COCs were classified into immature and mature groups based on morphological criteria after 24 hours of culture. Total RNA was extracted from both groups and CHI3L1 expression was quantified using quantitative real-time PCR (qRT-PCR). Western blot analysis was performed to assess CHI3L1 protein levels in both immature and mature oocytes.

Result: The study found a significant upregulation of CHI3L1 expression in mature COCs, with a 3-fold increase compared to immature COCs ($p < 0.05$). Western blot analysis confirmed the higher intensity of CHI3L1 protein in mature oocytes, correlating with the qRT-PCR results. These findings suggest that CHI3L1 plays a crucial role in the maturation of buffalo oocytes, potentially through its involvement in tissue remodelling and inflammatory responses within the COCs.

Key words: Buffalo, Chitinase-3-like protein 1, Cumulus-oocyte complex, *In vitro* maturation.

INTRODUCTION

Buffaloes (*Bubalus bubalis*) are vital to South Asia, especially in India, where they contribute significantly to the agricultural economy, providing food, employment and more than half of the country's total milk production. Due to their adaptability to India's hot, humid climate, buffaloes offer superior milk quality and versatility for meat, milkand draft power, making them economically valuable (Siddiky and Faruque, 2018). However, challenges like seasonal feed availability and reproductive inefficiencies affect their productivity. While advancements in assisted reproductive technologies (ART) like artificial insemination and cryopreservation have been made, *in vitro* embryo production (IVP) remains less efficient than in cattle, mainly due to low embryo survival rates and inadequate in vitro culture conditions (Balhara *et al*., 2022). Improving in vitro maturation (IVM) of oocytes is crucial for enhancing IVP success, as it prepares oocytes for fertilization and subsequent embryo development (Baruselli *et al*., 2023; Shahzad *et al*., 2023). Research has demonstrated the role of growth factors and hormonal supplements in improving IVM outcomes. Bera *et al*. (2024) found that adding epidermal growth factor and fibroblast growth factor to the maturation media improved cleavage rates and blastocyst formation in cattle oocytes. Similarly, Rajesh *et al*. (2020) reported that kisspeptin combined with FSH and LH significantly enhanced oocyte maturation in buffaloes. The cumulus-oocyte complex (COC), comprising an oocyte surrounded by cumulus cells, is essential for oocyte maturationand understanding the molecular mechanisms regulating this interaction is crucial for improving buffalo reproduction (Turathum *et al*., 2021; Moussa *et al*., 2018). Chitinase-3-Like Protein 1 (CHI3L1), or YKL-40, plays a key role in immune response, tissue remodelingand inflammation, despite lacking chitinase activity (Mizoguchi *et al*., 2024). CHI3L1 is involved in processes like epithelial-mesenchymal transition and immune regulation, as demonstrated in mammary epithelial cells, mouse model

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of mastitis and bovine tuberculosis (Anand *et al*., 2016, Breyne *et al*., 2018; Fang *et al*., 2020). While its role in other physiological contexts is well studied, the expression and function of CHI3L1 in buffalo COCs during IVM remain largely unexplored. This study aims to investigate the expression of CHI3L1 in buffalo COCs during IVM and explore its potential impact on oocyte competence and embryonic development. Insights gained could help improve IVM protocols and enhance buffalo reproductive efficiency.

MATERIALS AND METHODS

Sample collection

Buffalo ovaries were collected within 20-30 minutes post-slaughter at a local abattoir in Chennai, Tamil Nadu, India and transported in sterile saline supplemented with Penicillin (100 µg/mL) and Streptomycin (100 µg/mL), maintained at 30-35°C, with the time from collection to oocyte retrieval minimized to 2-3 hours. Upon arrival at the Centralized Embryo Biotechnology Unit located at Madhavaram Milk colony, Chennai, ovaries were thoroughly washed with sterile saline to remove blood and debris. Using a sterile surgical blade, multiple incisions were made on the ovarian surface to release cumulus-oocyte complexes (COC) into the Oocyte collection medium (114 mM NaCl, 3.2 mM KCl, 2.0 mM $NaHCO_3$, 0.4 mM NaH_2PO_4 (anhydrous), 10.0 mM sodium lactate (60% w/v), 0.5 mM $MgCl_2 \cdot 6H_2O$, 2.0 mM $CaCl_2 \cdot 2H_2O$, 10.0 mM HEPES, 0.5 mM sodium pyruvateand 5.6 mM glucose). The released COCs were carefully collected using a pipette under a zoom stereomicroscope (Nikon, Japan) and transferred to a fresh washing medium containing TCM-199 + 10% FBS + 0.81 mM Sodium Pyruvate + 50 µg/ml gentamicin solution. The oocytes were then screened and classified based on the presence of cumulus cells and cytoplasmic homogeneity, selecting only those with optimal characteristics for subsequent *in vitro* maturation. The experiments were conducted at the Centralized Embryo Biotechnology Unit, Department of Animal Biotechnology, Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University (TANUVAS), Chennai, Tamil Nadu, during the period from December 2023 to July 2024.

In vitro maturation (IVM)

The cumulus-oocyte complexes (COCs) were then washed multiple times in washing medium (TCM-199 + 10% FBS + 0.81 mM Sodium Pyruvate + 50 µg/ml gentamicin solution). After washing, 20-25 COCs were placed into 100 µl droplets of maturation medium (TCM-199 + 10% FBS + 5 µg/ml FSH +5 µg/ml luteinizing hormone (LH) + 1 µg/ml estradiol + 0.33 mM Sodium Pyruvate + 50 µg/ml gentamicin sulfate) overlaid with sterile mineral oil. These droplets were then incubated at 38.5°C with 5% CO2 for 24 hours to allow maturation. Following incubation, the oocytes were assessed for cumulus expansion which confirms successful maturation.

RNA extraction and qRT-PCR

Total RNA was extracted from both immature and mature cumulus-oocyte complexes (COC) using a commercial RNA extraction kit (RNeasy Mini Kit, Qiagen, Germany). The RNA concentration and purity were assessed using a spectrophotometer with a microvolume cuvette (Eppendorf BioSpectrometer, Eppendorf, Germany). Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using a PrimeScript™ RT Reagent Kit (Takara Bio Inc., Japan) according to the manufacturer's protocol. Quantitative real-time PCR (qRT-PCR) was performed to quantify CHI3L1 gene expression using a SYBR Green PCR Master Mix (SYBR® Premix Ex Taq™, Takara Bio Inc., Japan) on a CFX96 Touch Real-Time PCR Detectzion System (Bio-Rad Laboratories, USA). The 18S rRNA gene was used as the internal control or housekeeping gene for normalization. Primers for CHI3L1 were designed based on the buffalo CHI3L1 gene sequence available in GenBank (Table 1). The qRT-PCR reactions were carried out in a 20 µL reaction volume containing 10 µL of SYBR Green master mix, 1 µL of forward and reverse primers (10 µM each), 1 µL of cDNA templateand 8 µL of nuclease-free water. The thermal cycling conditions were as follows: initial denaturation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C (both CHI3L1 and 18SrRNA) for 15 secondsand extension at 72°C for 20 seconds. A melt curve analysis was performed at the end of each run to confirm the specificity of the amplification. Relative expression of the CHI3L1 gene in mature versus immature COCs was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Western blot analysis

Protein isolation from both immature and mature oocytes was performed by lysing 150 oocytes in RIPA buffer (Sigma, USA) supplemented with 1% protease inhibitor cocktail (Sigma, USA) and incubating on ice for 30 minutes. The lysates were mixed with SDS sample buffer, boiled for 5 minutes and subjected to separation on a 12% SDS-polyacrylamide gel. A protein ladder (PageRuler Prestained

Table 1: The primer sequences used in real-time PCR analysis.

Genes	Primer sequences	Accession number
CHI3L1	5'GGGTGGCTCAGACAGGTTTT3'(F)	NM_001290968.1
	5'TCCATCAAAGCCATGGGTCC3'(R)	
18SrRNA	5' AAGTCTTTGGGTTCCGGG 3'(F)	NR_036642.1
	5' GGACATCTAAGGGCATCACA 3'(R)	

Protein ladder 10 to 180kDa, Thermo Scientific, USA) was also loaded alongside the samples to serve as a molecular weight marker. The proteins were then transferred onto a PVDF membrane using a semi-dry transblot apparatus (GE, USA). The membrane was blocked with TBST containing 5% low-fat dry milk for 2 hours at room temperature. The membrane was incubated overnight at 4°C with a rabbit-raised YKL-40 primary antibody (Sino Biological, China), followed by washing and incubation with HRP-conjugated anti-rabbit IgG (Abcam, UK) for 2 hours at room temperature. After washing, the protein bands were visualized using a DAB substrate kit (Sigma, USA) and imaged using a suitable system.

Statistical analysis

The results were analysed by using Graph Pad Prism 5 (Graph Pad Software, San Diego, CA, USA). Real-time PCR data were expressed as mean ± Standard deviation (SD). Statistical significance was assessed using the student's t-test, with p-values<0.05 considered significant.

RESULTS AND DISCUSSION

In vitro maturation of buffalo oocytes

Oocytes were first recovered using the slicing method, where ovaries were sliced with a sterile surgical blade into a collection medium to release the cumulus-oocyte complexes (COCs). The recovered COCs were then graded based on the number of cumulus cell layers surrounding the oocyte. For in vitro maturation, oocytes that had a dense and intact cumulus cell layer, classified as A-grade, were selected. These A-grade oocytes, characterized by multiple layers of cumulus cells and a homogeneous cytoplasm, were then subjected to the IVM protocol Fig 1A. The Results from the in vitro maturation process revealed that most buffalo COCs efficiently completed maturation under the applied conditions. Specifically, the oocytes exhibited clear expansion of cumulus cells, indicating effective maturation. The high maturation rates and observed morphological changes, such as a homogeneous cytoplasm and well-expanded cumulus cells, confirmed that the IVM protocol successfully supported buffalo oocyte maturation Fig 1B.

Quantitative polymerase chain reaction analysis

Real-time quantitative PCR analysis of CHI3L1 expression in buffalo COCs showed significant upregulation of the gene in mature oocytes. The expression of CHI3L1 was measured relative to the housekeeping gene 18S ribosomal RNA (18S rRNA), serving as an internal control. Specifically, CHI3L1 expression was approximately three times higher in mature COCs compared to immature ones ($p<0.05$), indicating that CHI3L1 is upregulated during maturation Fig 2. The PCR amplification product of CHI3L1 was verified through agarose gel electrophoresis. A band corresponding to the expected molecular size of 392 bp is clearly seen in the agarose gel Fig 3, demonstrating that the primers designed for CHI3L1 amplification were functional.

Protein analysis

Western blot analysis confirmed that CHI3L1 protein bands were visible in both immature Fig 4, Lane B and mature oocytes Fig 4, Lane A, with an apparent molecular weight of approximately 40 kDa. The band corresponding to CHI3L1 was more intense in mature oocytes (Lane A) compared to immature oocytes (Lane B), indicating increased expression of CHI3L1 during oocyte maturation. This observation aligns with the qRT-PCR results, which also showed upregulation of CHI3L1 mRNA in mature oocytes, confirming that the increase in CHI3L1 at the transcript level is reflected at the protein level.

The upregulation of CHI3L1 in mature buffalo COCs emphasizes the likelihood of this protein playing a role in oocyte maturation. The study reports a 3-fold increase in CHI3L1 expression in mature oocytes compared to immature ones, suggesting the protein's involvement in critical processes such as tissue remodeling, inflammation and cellular proliferation, which are important during oocyte maturation. To the best of our knowledge, this is the first report regarding the expression of CHI3L1 in oocytes in general and specifically in buffalo. CHI3L1, also known as YKL-40, is a member of the glycoside hydrolase family 18 and has been extensively studied for its role in various physiological and pathological processes. Its function in tissue remodeling is particularly important for oocyte

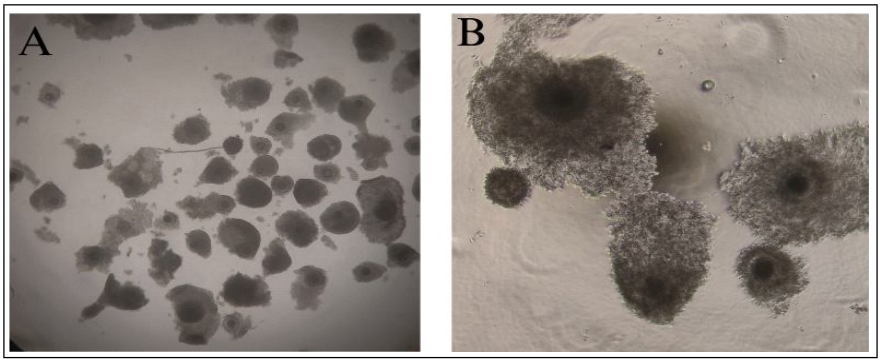


Fig 1: Representative Images of immature (A) and matured (B) buffalo oocytes.

maturation. Tissue remodeling, along with extracellular matrix (ECM) synthesis, is crucial for creating a supportive microenvironment for oocyte maturation. The significant upregulation of CHI3L1 observed in this study likely reflects its role in ECM remodeling, which is essential for successful follicle growth and oocyte quality. These findings align with those reported by Barbato *et al*. (2023), who highlighted the importance of ECM remodeling under dynamic culture conditions for improving follicle viability and oocyte maturation. Similarly, Grosbois *et al*. (2023) demonstrated that *in vitro* culture induces spatiotemporal remodeling of the human ovarian cortical ECM, emphasizing the role of its components-collagen and elastin-in follicle activation and growth. Besides its role in ECM remodeling, CHI3L1 also participates in mediating inflammatory responses, which are increasingly recognized as key to reproductive biology. Controlled inflammation can trigger and promote timely tissue remodeling and repair, both of which are required for oocyte maturation. CHI3L1's involvement in these processes is also documented in other contexts, particularly its anti-inflammatory role in synovial tissues during arthritis (Recklies *et al*., 2002). In the reproductive tract, regulated inflammatory responses are central to processes such as ovulation and the preparation of the endometrium for implantation (Espey, 1994; Critchley *et al*., 2020). The increased expression of CHI3L1 in mature oocytes suggests it might be involved in regulating these inflammatory processes within the COCs. CHI3L1's involvement in the TGF-β signaling pathway is primarily associated with cancer progression, where it promotes tumor growth and metastasis, as observed in studies on hepatocellular carcinoma (Qiu *et al*., 2018). However, the exact role of CHI3L1 in ovarian follicle development and oocyte maturation remains to be clearly defined. The TGF-β signaling pathway is known to regulate cumulus cell expansion and nuclear maturation, both essential for the successful maturation of oocytes in various species, including pigs, cowsand buffaloes (Doultani *et al*., 2022; Mahanta *et al*., 2018; Nagyova, 2012). Growth differentiation factors like GDF9, secreted by oocytes, have been found to interact with TGF-β signaling to control cumulus cell functions and oocyte maturation (Yu *et al*., 2016). This underscores the importance of TGF-â and related proteins in reproductive biology and suggests that CHI3L1, known to influence TGF-β signaling in other contexts, might similarly impact oocyte maturation, although direct evidence in this area is limited (Zhu *et al*., 2008; Gomez *et al*., 2012). As reported, CHI3L1 has been shown to activate the PI3K/AKT signaling pathway, which plays a crucial role in cell survival, metabolismand proliferation (Anand *et al*., 2016). This pathway is particularly important for oocyte maturation due to its ability to support metabolic activity and cell survival, both of which promote oocyte quality. CHI3L1 has been widely recognized for activating the PI3K/AKT pathway in various contexts, including cancer biology, where it enhances stem-like properties through Akt signaling during tumor progression. For instance, in ovarian cancer, CHI3L1 induces the Akt pathway and upregulates the expression of β-catenin and SOX2, critical transcription factors for maintaining stemness and resistance to apoptosis (Lin *et al*., 2019). This suggests that CHI3L1 might similarly modulate the PI3K/AKT pathway in oocytes, thereby influencing metabolic and survival pathways and playing a vital role in oocyte quality and maturation (Jiao *et al*., 2020; Kalous *et al*., 2023). In the study by Ozegowska *et al*. (2019), gene expression profiling in porcine granulosa cells highlighted the importance of cellular processes such as proliferation, migrationand adhesion. CHI3L1 was identified as one of the significantly upregulated genes during *in vitro* culture, indicating its potential role in cellular adhesion and motility,

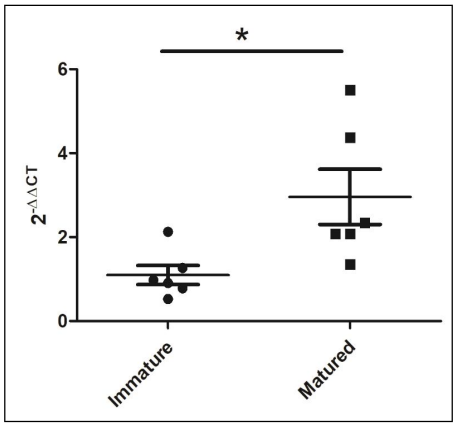
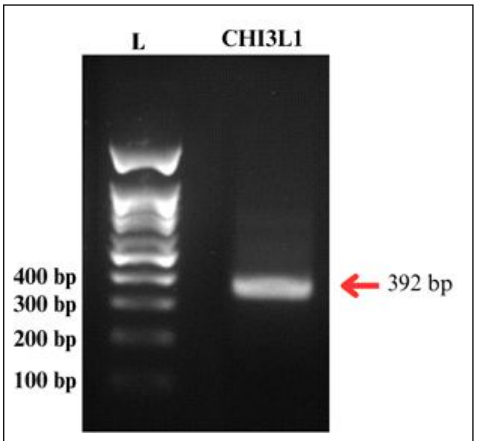
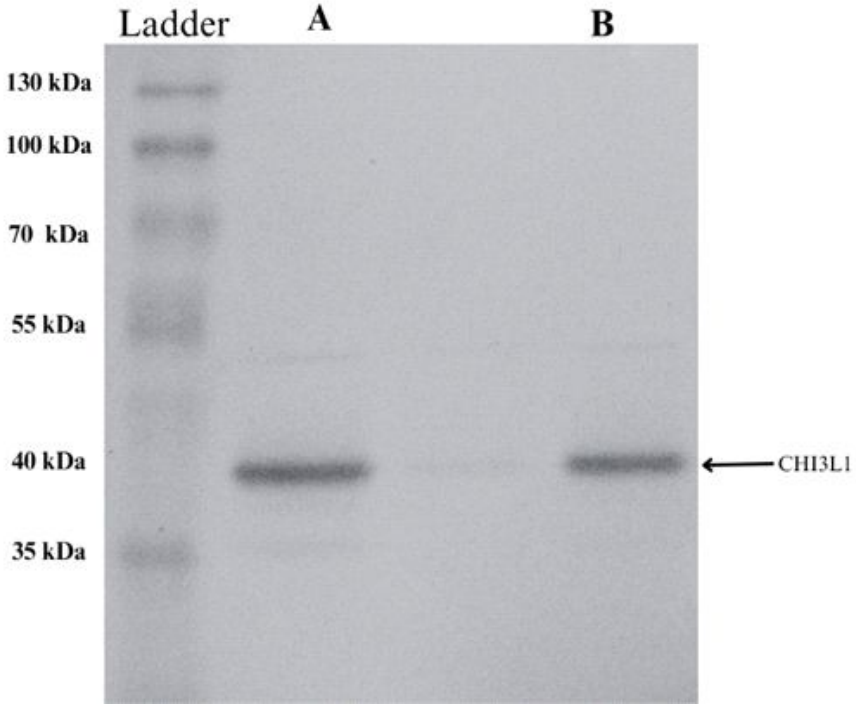


Fig 2: qPCR analysis revealed a 3-fold increase in CHI3L1 expression in Matured oocytes compared to Immature COC. n=6, *p(<0.05).



Lane L represents the DNA ladder with indicated base pair (bp) markers (100 bp, 200 bp, 300 bpand 400 bp). Lane CHI3L1 shows the amplification of the CHI3L1 gene, resulting in a specific band at approximately 392 bp, as indicated by the arrow.

Fig 3: Agarose gel electrophoresis of CHI3L1 PCR product.



A-Matured Oocyte. B- Immature oocyte. The arrow indicates a 40 kDa CHI3L1 in mature and immature oocytes.

Fig 4: Western Blot analysis for CHI3L1 in immature and matured oocyte lysate.

which are crucial for follicular development and oocyte quality. Furthermore, the expression of CHI3L1 in human embryonic stem cells (hESCs) and their differentiated progeny supports its role in early cell differentiation and commitment to specific lineages (Brochner *et al*., 2011). In hESCs, CHI3L1 is differentially expressed during the transition from pluripotent states to more differentiated ones, particularly under varying culture conditions such as oxygen tension and growth factors like bFGF. This suggests that CHI3L1's role in cell differentiation may be conserved across species and could extend to buffalo oocyte maturation. Moreover, the detection of CHI3L1 in cervicovaginal mucus suggests its possible role in the immune defense mechanisms of the reproductive tract, which could impact reproductive health and fertility (Maddison *et al*., 2017). This finding broadens the relevance of CHI3L1 in reproductive biology, indicating that it may play multiple roles in ensuring reproductive success. Several studies have also explored CHI3L1's involvement in estrogen regulation. For example, CHI3L1 is upregulated in the uterine fluid of mice with disrupted estrogen receptor signaling, suggesting a complex interplay between estrogen levels and CHI3L1 expression (Antonson *et al*., 2015). Although its precise role in reproduction under these conditions is not fully understood, its upregulation in estrogenic states could imply an indirect regulatory effect on oocyte maturation. These findings have important implications for reproductive biotechnology. Identifying CHI3L1 as a potential biomarker for oocyte maturity could serve as a valuable tool for selecting oocytes with higher developmental competence before IVM.

CONCLUSION

In conclusion, this study highlights the role of CHI3L1 in oocyte maturation and identifies it as a potential biomarker for oocyte quality. The upregulation of CHI3L1 in mature COCs suggests its critical role in the maturation process, likely through tissue remodelling, inflammationand protection against apoptosis. Future research should further explore the specific pathways through which CHI3L1 operates in oocyte maturation and investigate how this protein can be utilized to enhance reproductive efficiency in buffaloes and other livestock species.

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Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data

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