Transcriptional Level of Cumulus-Associated *GJA1*, *PTX3*, *PRSS35*, and *SERPINE2* Genes with Oocytes and Embryonic Development in Water Buffalo

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Abstract

Background:

In the present study, the potential of different groups of cumulus-oocyte complexes (COC's) for *in-vitro* maturation (IVM) and embryonic development was assessed in two groups of COC's of water buffalo. Further, the association of the expression pattern of cumulus-associated *GJA1*, *PTX3*, *PRSS35*, and *SERPINE2* genes and their effects on embryonic development was analyzed. Slaughterhouse-derived buffalo oocytes were graded into group A and B based on surrounding cumulus rings. Out of 1000 ovaries, an equal number of 410 COC's were taken in both the A and B groups. *In-vitro* maturation (IVM) was carried out using Slaughterhouse-derived buffalo epididymis. A remarkable degree of cumulus expansion was noticed in group A (92.68%) as compared to group B (81.25%) oocytes. On IVF and embryo culture, group A COC's produced a significantly higher rate of cleavage and blastocyst ($92.682 \pm 0.7179\%$ and $42.682 \pm 0.9683\%$) as compared to group B COC's ($85.365 \pm 0.7608\%$ and $31.707 \pm 0.9688\%$), respectively. The transcriptional analysis of cumulus-associated *GJA1*, *PTX3*, *PRSS35*, and *SERPINE2* genes expression by quantitative Real Time-PCR (qRT-PCR) revealed a significantly higher expression in group A as compared to group B COC's.

Result: It was revealed that oocytes having good cumulus mass had a higher developmental potential. Based on differential gene expression of cumulus-associated genes, different quality of COC's, and the resultant embryos after IVF, it was concluded that these genes could be used as a marker for predicting the developmental competence of the oocytes.

Conclusion: It concluded from the study that morphologically good quality of COC's had a higher developmental competence. Also, the differential expressions of cumulus-associated genes in cumulus cells and embryos, we can conclude that these genes could be used as marker genes for predicting the developmental competence of buffalo's oocytes.

Background

In-vitro maturation (IVM), *in-vitro* fertilization (IVF), and gamete cryopreservation technologies provide a practical means for producing a large number of bovine embryos at a low cost for research and commercial settings [1–3]. Various factors have been affected the successful In-Vitro production of buffalo embryos including a low number of follicles on the ovaries, sperm mortality, in-vitro laboratory conditions. [4–5], a poor recovery rate of the oocytes [6–7], and poor *in-vitro* fertilization efficiency [8–10]. In early embryo development, defining oocyte quality remains one of the most difficult challenges. Follicular growth and maturation are prerequisites to oocyte fertilization and subsequent early embryo development [8]. Among all the events involved in this process, those taking place precisely at the pre-ovulatory stage within the cumulus-oocyte-complexes (COC's) might offer new criteria for choosing embryos with the best development ability [11–13]. Previous studies emphasized the roles of cumulus mass in nurturing oocyte growth, gradual acquisition of oocyte developmental competence suggested

that cumulus cells metabolize the bulk of glucose consumed by the COC's, supplying metabolic intermediates like pyruvate, mainly through glycolysis, to the oocyte and regulating the oocyte gene transcription [14–16].

In the livestock sector, *in-vitro* fertilization (IVF) technology can be a useful tool for fruitful output in mammalian species like cows, buffaloes, goats, and pigs. The good qualities of cumulus cells (CC's) are very important for successful *in-vitro* fertilization. Oocyte cumulus undoubtedly plays an important role in oocyte quality and maturation but the eject mechanism behind this is still unknown [17]. Therefore, an extensive study is required to analyze whether the developmental potential of the COC's is associated with the expression of different cumulus genes in terms of maturation, fertilization, and embryonic development. Works of literature show that genes such as *GJA1*, *PRSS35*, *PTX3*, and *SERPINE2* play a crucial role in oocyte maturation, fertilization, and embryonic development [18–24]. However, literature elucidating these cumulus-associated gene expressions, their significance, and the effect of cumulus cell layer/mass toward the embryonic developmental process are scarce in livestock.

Cumulus-associated *GJA1* gene is the major gap junction *a1* protein or *connexin43*, essential for oocyte fertilization potential and embryo quality [18]. *PRSS35* gene belongs to the trypsin class of serine proteases, which is essential for the tissue remodeling and functions of the ovary during folliculogenesis and ovulation [19]. The gene *PTX3* (Pentraxin-related protein 3) is linked to extracellular matrix (ECM) hyaluronan and is a potentially reliable predictor of embryo developmental competence [20]. The *SERPINE2* (serine proteases E2) gene is considered a potential pregnancy biomarker [21] and is extensively expressed in reproductive tissues [22–24].

Our aim was, therefore, to evaluate the effects of cumulus cell mass on oocyte competence in terms of oocytes maturation and post-fertilization developments along with the expression of *GJA1*, *PTX3*, *PRSS35*, and *SERPINE2* genes in cumulus cells according to stages in oocyte maturation and embryonic developmental competence in slaughterhouse derived buffalo oocytes.

Methods

All the chemicals and media were purchased from Sigma-Aldrich Chemicals Company (St. Louis, Monsanto) and the disposable plastic wares were from Nunc (Roskilde, Denmark) unless otherwise mentioned.

Oocyte Aspiration And Grading

Under a total of 30 trials (Sample size calculated by using, $n = Z^2 \cdot P(P-1) / D^2$ formula), buffalo ovaries were collected from the large animal abattoir aseptically in normal saline solution (37°C, pH 7.0), supplemented with gentamicin (50 µg/ml). The ovaries were trimmed and washed 3-4 times in Dulbecco's phosphate buffer saline (1X, DPBS). This was followed by a quick wash of 30-40 seconds with 70% ethanol and finally rinsed with DPBS. Oocytes were aspirated from 6-8 mm ovarian follicles by follicular

aspiration method in a pre-warm (37°C) DPBS medium. The oocytes were washed 3-4 times in TCM-199 (washing media, Hyclone), supplemented with 7.5% (v/v) fetal bovine serum (FBS, Hyclone,) plus 50 mg/ml gentamicin. Recovered oocytes were grouped into 4 categories A, B, C, and D [25], following the morphology of cumulus mass and cytoplasmic appearance of oocytes. Group A oocytes had a 3-4 compact layer of cumulus mass with evenly granular homogenous cytoplasm, whereas group B oocytes had 2-3 layers of cumulus mass with evenly granular homogenous cytoplasm. The oocytes with complete or incomplete 1-2 layers of cumulus mass with irregular dark ooplasm, and no cumulus mass with irregular dark ooplasm, were categorized under the C and D group of oocytes, respectively (Fig. 1a). Only group A and B COC's were taken for the gene expression study. Further, for gene expression analysis, oocytes from both groups were divided into three subsequent stages *viz.*, cumulus cells of immature COC's, cumulus cells of matured COC's and fertilized early embryos.

In-vitro maturation (IVM)

In-vitro maturation medium containing TCM-199 Supplemented with 10µg/ml follicle-stimulating hormone (FSH), luteinizing hormone (LH), 1µg/ml estradiol, 7.5 percent (v/v) fetal bovine serum (FBS), 50 µg/ml gentamicin, and 0.8 mM/ml sodium pyruvate. It was used for *in-vitro* maturation of oocytes in groups of 30 oocytes per droplet. The droplets were overlaid with sterile pre-equilibrated mineral oil. The maturation process was carried out under a humidified atmosphere (99%) for 24 hours in a CO₂ incubator having a 5% CO₂ level at 37°C. The cumulus expansion and presence of the first polar body were observed under the inverted zoom microscope (Nikon, 100X), to assess the *in vitro* maturation of oocytes.

Sperm preparation for in-vitro fertilization (IVF) and embryo culture

Under 30 experimental trials, sperms were collected from abattoir-derived epididymis and processed under sterile conditions as described earlier for ovaries. Sperms were harvested from tubules of epididymis with a fine and gentle incision with a sterile surgical blade and collected in a sterile tube containing 10 ml of Bracket and Oliphant (BO) media, supplemented with 1mM caffeine sodium benzoate. The sperm concentration, morphology, and gross motility were observed under the inverted microscope (Nikon, 100X). For *in-vitro* fertilization, the sperms were prepared and capacitated for matured oocytes as described by Jeena *et al* 2018 [26]. In short, exudate sperms suspension with BO media was centrifuged at 168G for 10 min and the supernatant was discarded. This step was repeated twice. The pellet was resuspended in 5ml of BO fertilization media fortified with 1% BSA plus 50 mg/ml heparin and centrifuged at 168 G for 5 min. The harvested pellet was re-suspended in 1ml BO fertilization media and centrifuged at 168 G for 1min. The pellet was loosened with a fine bore pasture pipette and kept inside the CO₂ incubator for 30 min for capacitation and to allow good quality sperms to swim up. At the same time 30 matured oocytes from both groups A and B were transferred into a pre-equilibrated (38.5°C) 50µl BO fertilization droplet overlaid with mineral oil. After 30 min capacitated sperms with a final concentration of 10⁶ were taken from the top layer of sperm suspension and co-incubated with oocytes at 5 percent CO₂ at 38.5°C temperature under a humidified atmosphere for 18 h (Fig. 1b). After 18 h of co-

incubation, oocytes were denuded with 1 percent of hyaluronidase. The presumptive zygotes (Fig. 1c and Fig. 1d) were cultured in RVCL media (Research Vitro Cleave Medium, COOK, Australia). Each group of embryos were assessed for their embryonic developmental stages as cleavage (Fig. 1e), 4 cells (Fig. 1f), compact morula (Fig. 1g), and blastocyst development (Fig. 1h).

Primer designing

The Primers for *β-actin*, *PTX3*, *PRSS35*, and *SERPINE2* were designed by using online software PRIMER EXPRESS 3.0. The *GJA1* gene primer was taken as a reference primer. Primer sequence specificity was checked by an online available database from BLAST, NCBI (Tab. 1).

Table 1
Details of qRT-PCR (quantitative Real Time-PCR) primers

S. No.	Gene name	Primer Sequence (5'-3')	Amplicon size
1.	<i>β-actin</i> (R)	CACCCAGCACAATGAAGA	118bp
1	<i>β-actin</i> (F)	TGCTTGCTGATCCACATC	
3.	<i>SERPINE2</i> (F)	AAGGGTTTGTGGAGATCACG	170bp
	<i>SERPINE2</i> (R)	TAGGGCAGCTCGATGAAGTT	
5.	<i>PRSS35</i> (F)	CGACTACATCAAAGGCAGCA	179bp
	<i>PRSS35</i> (R)	ATCCCTTTCTCCTCCTTCCA	
7.	<i>PTX3</i> (F)	CCATGCGTTCCAAGAAGATT	217bp
	<i>PTX3</i> (R)	GCGACCAGTCTGTTTTCTC	
9.	<i>GJA1</i> (F)	CCTGCCGCAATTACAACAAACAAG	425bp ^[25]
	<i>GJA1</i> (R)	ATCCCTAACACCCCCAATGAACCA	
F* forward primer; R* reverse primer			

Gene expression level of cumulus-associated GJA1, PTX3, PRSS35, and SERPINE2 genes

Cumulus-oocyte complexes (COC's) were denuded in 0.1 percent hyaluronidase for 10 min with a gentle vortex to remove the cumulus cells. The total RNA was extracted by the Trizol method from cumulus cells of both groups (A and B), and also from their subsequent stages of immature cumulus cells of COC's, matured COC's, and fertilized early embryos. A total of 30 COC's from both groups were taken for RNA extraction. cDNA was prepared by using the first-strand cDNA synthesis kit (Fermentas, K1622) from an equal amount of total RNA.

qRT-PCR was performed with a total volume of 20µl containing cDNA (1µl), 1pico- mole of reverse and forward primer (Table 1), and 10µl of 2X SYBER green master mix (Applied Biosciences). All reactions were performed in triplicate. The relative quantification of mRNA of each gene was evaluated by qRT-PCR (Applied Biosystems, 7300) using SYBR green chemistry. The relative gene expression analysis of *GJA1*, *PTX3*, *PRSS35*, and *SERPINE2* genes was analyzed by using Livak and Schmittgen [27] formulation *i.e.*, comparative $2^{-\Delta\Delta Ct}$ method. The housekeeping gene β -actin was used as an endogenous loading control to normalize the relative gene expression. Immature COC's of group A oocyte were taken as a referral control (1.0) for the expression analysis of cumulus-associated *GJA1*, *PTX3*, *PRSS35*, and *SERPINE2* genes in both the groups. The purity of amplified products was checked by Ethidium Bromide (EtBr, 0.5µg/ml) stained with 1.8 percent agarose gel electrophoreses.

Statistical analysis

The percent data of embryonic development were transformed by arcsine transformation ($\log_N$) and subjected to analysis of variance by Hierarchical analysis of variance design, and the mean differences were compared by Duncan's Multiple Range Test ($P < 0.05$).

Results

In-vitro maturation (IVM) of abattoir-derived buffalo oocytes

Under 30 experimental trials, a total of 2070 COC's were retrieved from 1000 slaughterhouse-derived ovaries, out of which 550 were from group A, 800 from group B (Table 2), and the rest of 720 were from group C and D. Only good quality COC's of group A and B were taken for *in-vitro* maturation and in *in-vitro* fertilization. The analysis of variance of the data on embryonic development (Table 3) revealed a highly significant ($P < 0.01$) between-group difference (A and B) for all the embryonic stages. However, there was no significant difference ($P > 0.01$) between the day of the experiment (bull effect), indicating that epididymal semen collected on a different day did not significantly affect embryonic development. Further, the embryonic development stages within the group differed significantly, shown in Table 3.

It was observed that the *in-vitro* maturation rate of group A was substantially higher as compared to group B oocytes, in terms of first polar body extrusion (Table 2). The cleavage (2 cells), 8 cells, morula and blastocyst development rate (Table 4) in group B (85.365 ± 0.7608 , 65.853 ± 0.7608 , 51.219 ± 0.9575 and $31.707 \pm 0.9688\%$) COC's were found significantly lower ($P \leq 0.05$) compared to group A (92.682 ± 0.7179 , 78.804 ± 0.6878 , 59.756 ± 0.9466 and $42.682 \pm 0.9683\%$) in respect of all the embryonic development stages.

Table 2
In-vitro maturation of group A and B of oocytes.

Ovaries collected from buffalo slaughterhouse	Oocytes aspirated	Grading of oocytes taken for study	No. of oocytes set for IVM	Oocytes showing polar body
1000	2070	Group A	550 (26.57%)	508 (92.36%)
	(2.07 oocytes ovary)	Group B	800 (38.64%)	650 (81.25%)
Values within parentheses indicate mean±SE%				

Table 3
Analysis of variance for embryonic development.

Source of variation	Degree of freedom	Mean Square value
Between Group	1	0.535**
Between the day of experiment (bull effect)	28	0.401 ^{NS}
Between EDS within group	3	23.812**
Error	101	0.003
EDS-Embryonic development stage; ** Significant (P<0.01), NS- Non-Significant.		

Table 4
Mean embryonic development percentage in group A and B *in-vitro* fertilized oocytes.

No. of oocytes in each group	Embryonic development stages (Mean±SE)			
	2-4 cells (%)	8-16 cells	Morula (%)	Blastocyst (%)
Group A - 410 fertilized oocytes	380	320	245	175
	(92.682±0.7179) ^a	(78.804±0.6878) ^a	(59.756±0.9466) ^a	(42.682±0.9683) ^a
Group B - 410 fertilized oocytes	350	270	220	130
	(85.365±0.7608) ^b	(65.853±0.7608) ^b	(51.219±0.9575) ^b	(31.707±0.9688) ^b
Value with different superscripts within column differs significantly (P≤0.05).				

Transcriptional level GJA1, PTX3, PRSS35, and SERPINE2 genes in group A and B oocytes

The immature COC's of group A oocytes were taken as a referral control (1.0) for the expression analysis of *GJA1*, *PTX3*, *PRSS35*, and *SERPINE2* genes in both group A and B. Further, both the groups were divided into their three subsequent stages of cumulus cell of immature COC's, cumulus cells of matured COC's and fertilized early embryos.

Cumulus-associated GJA1 gene expression and oocyte integrity

The relative expression of the *GJA1* gene was found significantly ($P \leq 0.05$) higher in cumulus cells of immature COC's (1.0) compared to matured cumulus cells of COC's (0.74) and in the fertilized early embryos (0.22) of group A. The expression pattern was also significantly lower in group B immature cumulus cells of COC's (0.82), matured cumulus cells of COC's (0.42), and the fertilized early embryos (0.20) compared to group A (Fig. 2a). The present study revealed that the *GJA1* gene was strongly associated with immature cumulus cells of COC's, and the abundance of this gene was reduced significantly towards the progression of developmental stages till fertilization (shown in Fig. 2a-b).

Figure 2a Relative quantification of the *GJA1* gene in cumulus cells of COC's and *in-vitro* fertilized group A and group B oocytes. RC GA-IM - Referral Control of group A, cumulus cells of immature COC's. GB-IM - Group B, cumulus cells immature COC's. GA-M - Group A, cumulus of matured COC's. GB-M - Group B, cumulus of matured COC's. GA-F - Group A, fertilized early embryos. GB-F - Group B, fertilized early embryos. **b** Transcriptional level of *GJA1* gene. Lane 1 - Endogenous control β *actin*. Lane 5 = 100bp ladder. Lane 2, 3, 4 - Cumulus cells of immature COC's, cumulus cells of Matured COC's and fertilized early embryos of group A. Lane 6, 7, 8 - Cumulus cells of immature COC's, cumulus cells of matured COC's and fertilized early embryos of group B.

Cumulus-expressed PRSS35 and PTX3 mRNA levels correlated with oocyte maturation

It was observed that *PRSS35* and *PTX3* had significantly ($P \leq 0.05$) higher expression levels in matured cumulus cells of COC's of both group A (1.3 and 1.3) and group B (1.2 and 1.1). Whereas, the expression levels of *PRSS35* and *PTX3* were significantly lower ($P \leq 0.05$) in fertilized early embryos of both group A (0.33 and 0.52) and B (0.22 and 0.24). Similar results were observed in immature cumulus cells of COC's in group A (1.0) and B (0.91 and 0.80). The results showed that the relative transcripts of *PRSS35* and *PTX3* genes increased significantly in *in-vitro* matured stages, but were reduced markedly in fertilized early embryos irrespective of oocyte groups (Fig. 3a-b and 4a-b).

Figure 4a Relative quantification of the *PTX3* gene in cumulus cells of COC's and *in-vitro* fertilized group A and group B oocytes. RC GA-IM - Referral Control of group A, cumulus cells of immature COC's. GB-IM - Group B, cumulus cells immature COC's. GA-M - Group A, cumulus of matured COC's. GB-M - Group B, cumulus of matured COC's. GA-F - Group A, fertilized early embryos. GB-F - Group B, fertilized early embryos. **b** Transcriptional level of *PTX3* gene. Lane 1 - Endogenous control β *actin*. Lane 5 = 100bp ladder. Lane 2, 3, 4 - Cumulus cells of immature COC's, cumulus cells of matured COC's and fertilized early embryos of group A. Lane 6, 7, 8 - Cumulus cells of immature COC's, cumulus cells of matured COC's and fertilized early embryos of group B.

mRNA expression pattern of SERPINE2 gene in cumulus cells associated with fertilization and embryonic development:

The results showed that the relative expression of the *SERPINE2* gene was significantly higher ($P \leq 0.05$) in fertilized early embryos of both group A (1.6) and B (1.08). As shown in Fig. 5a, the *SERPINE2* gene expression was significantly lower ($P \leq 0.05$) in matured oocytes of both groups A (0.59) and B (0.46)

along with immature group B (0.79) and group A (1.0) oocytes. The expression level of the *SERPINE2* gene in embryos produced by *in-vitro* fertilization was significantly higher in fertilized early embryos than the cumulus cells of immature and matured COC's (Fig. 5a-b).

Figure 5a Relative quantification of the *SERPINE2* gene in cumulus cells of COC's and *in-vitro* fertilized group A and group B oocytes. RC GA-IM - Referral Control of group A, cumulus cells of immature COC's. GB-IM - Group B, cumulus cells immature of COC's. GA-M - Group A, cumulus of matured COC's. GB-M - Group B, cumulus of matured COC's. GA-F - Group A, fertilized early embryos. GB-F - Group B, fertilized early embryos. **b** Transcriptional level of *SERPINE2* gene. Lane 1 - Endogenous control β *actin*. Lane 5 = 100bp ladder. Lane 2, 3, 4 - Cumulus cells of immature COC's, cumulus cells, of matured COC's and fertilized early embryos of group A. Lane 6, 7, 8 - Cumulus cells of immature COC's, cumulus cells, of matured COC's and fertilized early embryos of group B.

The overall expression pattern of cumulus-associated *GJA1*, *PRSS35*, *PTX3*, and *SERPINE2* genes at their subsequent developmental stages in both groups were analyzed collectively. The expressions of these genes were found significantly higher in group A compared to group B (Fig S1). The amplified products were confirmed by amplification plot, melting curve (Fig. 6), and agarose gel electrophoresis. The products were identified by their specific band size (*GJA1*-425bp, *SERPINE2*-170bp, *PRSS35*-179bp, and *PTX3*-217bp) as shown in Fig. 2b, 3b, 4b, and Fig. 5b.

Discussion

Cumulus cells (CC's) originating from undifferentiated granulosa cells (GC's) differentiate in mural granulosa cells (MGC's) and CC's during antrum formation in the follicle by the distribution of location. [5–6]. *GJA1* is the major isoform of connexins between GCs (granulosa cells): MGCs–MGCs (mural granulosa cells) and MGCs–CCs (cumulus cells) [12]. Gap junctions transmit nutrients and small molecules such as ions, metabolites, amino acids, and intracellular signaling molecules from GCs to oocytes via CCs [18–19]. We observed the expression level of the *GJA1* gene in immature cumulus cells of COC's was significantly higher as compared to matured cumulus cells of COC's and fertilized oocytes of both groups (A & B, Fig. 2a-b). A similar type of study was reported by Edry and workers, they found *GJA1*-mediated gap junctional communication regulates oocyte meiosis resumption, and lower levels of *GJA1* in cumulus cells are beneficial for oocyte maturation [28]. While Feuerstein and Mishra reported contrasting results wherein relative expression of the *GJA1* gene was significantly lower in immature cumulus cells of COC's [29–30]. However, *GJA1* expression in cumulus cells surrounding the matured oocytes did not show any difference in developing embryos with good or poor morphology [29]. Hasegawa reported a significantly lower expression of *GJA1* for embryos with good morphology [12]. Similarly, we revealed significantly lower expression of the *GJA1* gene was observed in fertilized oocytes and matured cumulus cells of COC's.

PRSS35 belongs to the trypsin class of serine proteases, essential for follicular growth, ovulation, as well as for luteal formation and regression. *PRSS35* gene expression was localized in theca cells of pre-antral follicles, the theca and granulosa cells of pre-ovulatory, ovulatory follicles, and developing corpus luteum [15, 19, 31]. Whereas, the *PTX3* gene is localized in the cumulus matrix and plays a crucial role in cumulus expansion. Various cumulus proteins linked to extracellular matrix hyaluronan, are required for regulating cumulus integrity, which ensure cumulus expansion and oocyte maturation [14–15, 18, 20, 32–35]. A previous study reported *PRSS35* and *PTX3* mRNA levels are associated with oocyte interiority and fertilization potential [15].

Our data revealed that *PRSS35* and *PTX3* mRNA levels are associated with the oocyte maturation potential (Fig. 2). The higher expression of *PRSS35* and *PTX3* genes were observed in cumulus cells of COC's of matured oocytes of both groups (A & B, Fig. 3a-b & Fig. 4a-b). However, it was found significantly lower in cumulus cells of immature COC's and fertilized oocytes. Li et al [15] reported a relationship between *PRSS35* expression and oocyte competence. While the expression of *PTX3* has been reported to be significantly lower in cumulus cells from immature oocytes than in those from mature oocytes, which was found similar to our study. Similarly, Huang [32] demonstrated that the expression level of the *PRSS35* gene plays an important role in oocyte nuclear maturation and gaining developmental competence. According to Miyakoshi the *PRSS35* mRNA level increased at the time of ovulation and remained elevated in the developing corpus luteum [36]. Although the scientific literature regarding the association of the *PRSS35* and *PTX3* genes with the oocyte and embryo developments is less in livestock. However, some studies have been reported in a few species like mouse and human oocytes and embryos.

Salustri and co-workers [34] reported the infertility of *PTX3* null mice was associated with severe abnormalities of the cumulus ionophores and failure of *in-vivo*, but not *in-vitro* oocyte fertilization. Zhang [20] reported that the expression of the *PTX3* gene in cumulus cells was indicative of oocyte and embryo quality. However, Diao and co-workers reported the *PRSS35*-null mice to have no defects in female fertility, suggesting that the gene is non-functional for murine fertility as well as in embryonic development. Also, he did not detect any compensatory up-regulation of other proteases reported in the uterus; but, the expression of other protease-related genes cannot be ruled out [31]. However, this remains unclear in the case of mammals. We found the higher expression of *PRSS35* and *PTX3* genes in cumulus cells of matured COC's had a beneficial effect on oocyte competence and embryonic development.

SERPINE2 is a Serpin peptidase inhibitor (clade E, member), which inhibits urokinase-type plasminogen activator (PLAU) and tissue-type plasminogen activator. These activators are associated with many types of reproductive processes, e.g., ovulation, embryonic development, and embryo implantation [22–24]. Hamel et al, reported the *SERPINE2* mRNA levels in granulosa cells have been suggested to be a potential pregnancy biomarker [21].

Our data revealed that *SERPINE2* mRNA levels are associated with oocyte fertilization potential.

We found the expression of this gene was higher in fertilized oocytes of both groups (A & B, Fig. 5a-b). However, the expression of the *SERPINE2* gene was lower in cumulus cells of immature and matured COC's (Fig. 5a). Hamel and co-workers [13] also reported the *SERPINE2* gene to be considered as a potential pregnancy biomarker and extensively expressed in reproductive tissues, the placenta, and the uterus [22, 31]. Sadeesh *et al*, reported the silencing of *SERPINE2* expression using small interfering RNAs or blockage of *SERPINE2* protein using a specific antibody did not affect oocyte maturation [2]. However, in a mouse, higher levels of *SERPINE2* were demonstrated to impair cumulus expansion and oocyte maturation [19, 23, 37–38]. Li and coworkers reported the higher *SERPINE2* expression levels were detected in cumulus cells of human immature oocytes than in those of mature oocytes [15]. According to our data, we found the higher expression of the *SERPINE2* gene in fertilized oocytes suggested its significant role in fertilization and embryonic development.

The results of the present study showed that in all the cases, the expressions of cumulus-associated genes in group A were significantly up-regulated as compared to group B (supplementary Figure S1). The higher expression of these genes in group A COC's can be related to higher embryonic development compared to group B COC's showing down-regulation of all these genes compared to group A COC's. It might be concluded that the significantly higher expression of the *GJA1* gene in cumulus cells of immature COC's along with *PRSS35* and *PTX3* genes in cumulus cells of matured COC's have a positive impact on oocyte developments. Similarly, a higher expression of the *SERPINE2* gene in fertilized early embryos may have beneficial effects on oocyte fertilization and embryonic development in buffalo.

We found some contradictory results from a previous study which was in human embryos, where the study reported the *GJA1* and *SERPINE2* represent potential gene markers associated with oocyte maturation and *PRSS35* may be correlated with oocyte fertilization potential [15, 39]. However, our study model was buffalo's embryos.

Conclusion

The selection of embryos with higher developmental potential has been one of the major factors in assisted reproductive technology (ART). Because, cumulus cells play a very important role in oocyte integrity and embryonic development. Although cumulus gene expression may represent a promising method compared with the currently used morphology-based method, more investigations are warranted. Thus, a prospective larger cohort study or the use of SET cumulus samples remains necessary to clarify the effectiveness. However, it was concluded from our study the morphologically good quality of COC's had a higher developmental potential in terms of maturation, fertilization, and embryonic development as compared to oocytes having poor quality of COC's (in both A & B groups). Based on molecular analysis of differential expressions of cumulus-associated genes we found the *GJA1* gene associated with immature oocyte integrity, *PTX3* and *PRSS35* represent gene markers potentially associated with oocyte maturation, and *SERPINE2* may be correlated with oocyte fertilization potential. In summary, *GJA1*, *PRSS35*, *PTX3*, and *SERPINE2* in cumulus cells of different quality of oocytes and their resultant embryos after IVF, we can

conclude these genes could be used as biomarkers for predicting the developmental competence of buffalo's oocytes.

Declarations

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Conflicts of interest/Competing interests: The authors declare that they have no Conflicts of interest.

Ethics approval: There was no need for ethical approval for this study. Slaughterhouse-derived ovaries and epididymis were used for *in-vitro* transcriptional analysis of cumulus-associated genes. No live animals were used.

Consent to Participate (Ethics): Not needed. In the in-vitro study, no live animals were used.

Consent to Publish (Ethics): Not needed. In the in-vitro study, no live animals were used.

Authors' contributions: Lalit Mohan Jeena wrote the manuscript. Bikash Chandra Sarkhel and Dharmendra Kumar helped in statistical analysis. Sandeep Rahangdale and Ajit Pratap Singh arranged the figures and references as per journal guidelines.

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Figures

Figure 1

Different developmental stages of buffalo embryo. **a** *In-vitro* maturation of different grades of the oocyte (100X). **b** Co-incubated of capacitated sperms with matured oocytes (100X). **c** Pro-nucleus after fertilization (2PN, indicated with a red arrow, 200X). **d** Secondary polar body (red arrow, 200X). **e** Cleavage (200X). **f** 4-Cells (200X). **g** Compact morula (200X). **h** Blastocyst (100X).

Statistically significant ($P \leq 0.05$) Bar depicts Mean $\pm$ SE

Figure 2

a Relative quantification of the *GJA1* gene in cumulus cells of COC's and *in-vitro* fertilized group A and group B oocytes. RC GA-IM - Referral Control of group A, cumulus cells of immature COC's. GB-IM - Group B, cumulus cells immature COC's. GA-M - Group A, cumulus of matured COC's. GB-M - Group B, cumulus of matured COC's. GA-F - Group A, fertilized early embryos. GB-F - Group B, fertilized early embryos. **b** Transcriptional level of *GJA1* gene. Lane 1 - Endogenous control *β actin*. Lane 5 = 100bp ladder. Lane 2, 3, 4 - Cumulus cells of immature COC's, cumulus cells of Matured COC's and fertilized early embryos of group A. Lane 6, 7, 8 - Cumulus cells of immature COC's, cumulus cells of matured COC's and fertilized early embryos of group B.

Statistically significant ($P \leq 0.05$) Bar depicts Mean $\pm$ SE

Figure 3

a Relative quantification of the *PRSS35* gene cumulus cells of COC's and *in-vitro* fertilized group A and group B oocytes. RC GA-IM - Referral Control of group A, cumulus cells of immature COC's. GB-IM - Group B, cumulus cells immature COC's. GA-M - Group A, cumulus of matured COC's. GB-M - Group B, cumulus of matured COC's. GA-F - Group A, fertilized early embryos. GB-F - Group B, fertilized early embryos. **b** Transcriptional level of *PRSS35* gene. Lane 1 - Endogenous control *β actin*. Lane 5 = 100bp ladder. Lane 2, 3, 4 - Cumulus cells of immature COC's, cumulus cells of matured COC's and fertilized early embryos of group A. Lane 6, 7, 8 - Cumulus cells of immature COC's, cumulus cells of matured COC's and fertilized early embryos of group B.

Statistically significant ($P \leq 0.05$) Bar depicts Mean $\pm$ SE

Figure 4

a Relative quantification of the *PTX3* gene in cumulus cells of COC's and *in-vitro* fertilized group A and group B oocytes. RC GA-IM - Referral Control of group A, cumulus cells of immature COC's. GB-IM - Group B, cumulus cells immature COC's. GA-M - Group A, cumulus of matured COC's. GB-M - Group B, cumulus of matured COC's. GA-F - Group A, fertilized early embryos. GB-F - Group B, fertilized early embryos. **b** Transcriptional level of *PTX3* gene. Lane 1 - Endogenous control β *actin*. Lane 5 = 100bp ladder. Lane 2, 3, 4 - Cumulus cells of immature COC's, cumulus cells of matured COC's and fertilized early embryos of group A. Lane 6, 7, 8 - Cumulus cells of immature COC's, cumulus cells of matured COC's and fertilized early embryos of group B.

Statistically significant ($P \leq 0.05$) Bar depicts Mean $\pm$ SE

Figure 5

a Relative quantification of the *SERPINE2* gene in cumulus cells of COC's and *in-vitro* fertilized group A and group B oocytes. RC GA-IM - Referral Control of group A, cumulus cells of immature COC's. GB-IM - Group B, cumulus cells immature of COC's. GA-M - Group A, cumulus of matured COC's. GB-M - Group B, cumulus of matured COC's. GA-F - Group A, fertilized early embryos. GB-F - Group B, fertilized early embryos. **b** Transcriptional level of *SERPINE2* gene. Lane 1 - Endogenous control β *actin*. Lane 5 = 100bp ladder. Lane 2, 3, 4 - Cumulus cells of immature COC's, cumulus cells, of matured COC's and fertilized early embryos of group A. Lane 6, 7, 8 - Cumulus cells of immature COC's, cumulus cells, of matured COC's and fertilized early embryos of group B.

Figure 6

Amplification plot and melting curve graph of *GJA1*, *PRSS35*, *PTX3* and *SERPINE2* genes.

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