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Cytoplasmic Darkness and PDIA3 Expression as Morphological and Molecular Indicators of Bovine Oocyte Competence for In Vitro Embryo Production

Sohanug Mehedi†, Rajosree Das†, Tasmi Binta Reza, Iffat Nur Jahan, Md. Ruhul Amin and Md. Munir Hossain*

Department of Animal Breeding and Genetics, Faculty of Animal Husbandry, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh.

†The authors contributed equally.

***Corresponding author:** Dr. Md. Munir Hossain; E-mail: mmhossain@bau.edu.bd

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ABSTRACT

In vitro embryo production (IVP) is a key biotechnology for accelerating genetic improvement in cattle. Oocyte quality is the primary determinant of IVP success. Among non-invasive selection markers, cytoplasmic coloration-particularly dark-colored cytoplasm (DCC) has been proposed as a visual indicator of lipid content and metabolic competence. This study evaluated the developmental potential of bovine oocytes based on cytoplasmic appearance and validated their competence through PDIA3 gene expression. Cumulus-oocyte complexes (COCs) collected from slaughterhouse ovaries were classified into dark-colored cytoplasm (DCC) and light-colored cytoplasm (LCC) groups and subjected to in vitro maturation (IVM), fertilization (IVF), and culture (IVC). DCC oocytes exhibited significantly greater increases in oocyte diameter (11.60 ± 0.55 µm vs. 4.68 ± 0.31 µm; $p = 0.0004$) and cumulus expansion (225.8 ± 17.0 µm vs. 142.8 ± 15.1 µm; $p < 0.001$). Developmental outcomes were also superior in the DCC group, with fertilization (84.53 ± 2.49% vs. 67.80 ± 5.37%; $p = 0.015$), cleavage (84.53 ± 2.49% vs. 67.80 ± 5.37%; $p = 0.017$), and blastocyst formation rates (37.39 ± 3.03% vs. 19.41 ± 2.39%; $p = 0.0034$) significantly higher than in the LCC group. To validate these phenotypic differences, PDIA3 expression was analyzed in germinal vesicle (GV) and metaphase II (MII) oocytes. DCC-derived oocytes showed significantly elevated PDIA3 transcript levels, strengthening the link between cytoplasmic coloration and molecular competence. These findings establish cytoplasmic darkness, is a practical indicator of oocyte quality, and PDIA3 expression validates this morphological assessment at the molecular level.

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Introduction

The application of in vitro embryo production (IVP) has become a cornerstone biotechnology in bovine reproduction, facilitating accelerated genetic improvement by enabling the mass propagation of elite female germplasm. Through IVP, multiple embryos can be generated from a single donor cow during one estrous cycle, thus enhancing the dissemination of superior genotypes, conserving genetic resources of endangered breeds, and increasing reproductive efficiency in both commercial and smallholder systems. Despite its growing adoption and integration with genomic selection strategies, the overall efficiency of IVP remains suboptimal, with blastocyst formation rates typically ranging from 30–40% under laboratory conditions (de Wit and Kruip, 2001; Nagano et al., 2006). Among the various biological and technical factors influencing IVP outcomes, the intrinsic quality of the oocyte is widely recognized as the most critical determinant of subsequent embryonic development and successful implantation.

Oocyte developmental competence is acquired through a complex interplay of nuclear and cytoplasmic maturation processes. While nuclear maturation is relatively straightforward to monitor via polar body extrusion or chromatin configuration, cytoplasmic maturation encompasses more subtle and multifaceted subcellular changes. These include the redistribution of mitochondria and cortical granules, the accumulation of transcripts and proteins essential for early embryonic development, and the modulation of organelle function to support fertilization and zygotic genome activation. Lipid droplets within the ooplasm, composed primarily of triglycerides, serve as crucial energy reservoirs that support mitochondrial β-oxidation, ATP synthesis, redox balance, and resilience against oxidative stress during preimplantation development (Torner et al., 2004; McEvoy et al., 2000; Jeong et al., 2009).

In resource-limited settings where molecular or biochemical profiling of oocytes is impractical, visual morphological assessment remains the primary means of oocyte selection. Traditional criteria such as cumulus cell integrity, cytoplasmic granularity, zona pellucida morphology, and oocyte size have been widely used. Among these, cytoplasmic coloration specifically, the degree of pigmentation observed under a stereomicroscope has emerged as a simple yet informative parameter. Oocytes with dark-colored cytoplasm (DCC) are often presumed to have higher lipid and mitochondrial content, correlating with increased metabolic activity and developmental potential, whereas light-colored cytoplasm (LCC) may reflect cytoplasmic immaturity and reduced competence (Nagano et al., 2006; Ebner et al., 2003). However, despite its practicality, the predictive power of cytoplasmic coloration has rarely been validated at the molecular level.

To address this gap, we introduced an additional molecular criterion into oocyte evaluation: the expression of protein disulfide isomerase A3 (PDIA3). PDIA3 is a multifunctional endoplasmic reticulum chaperone involved in oxidative protein folding, redox regulation, and early post-fertilization events such as sperm chromatin decondensation (Jingyu Li et al., 2014; Payer et al., 2003). Its expression has been previously associated with oocyte quality and early embryo development in other mammalian models, including pigs and mice (Barros et al., 2019; Catala et al., 2011), but its role in bovine oocytes remains largely unexplored. Understanding whether PDIA3 expression patterns align with cytoplasmic color and IVP outcomes could provide crucial mechanistic insights and strengthen the validity of non-invasive selection protocols.

Therefore, the objectives of this study were twofold. First, we sought to evaluate the morphological and developmental differences between bovine oocytes categorized by cytoplasmic coloration into DCC and LCC groups, assessing parameters including oocyte diameter, cumulus expansion, fertilization rate, cleavage rate, and blastocyst yield. Second, we investigated the expression of PDIA3 in oocytes at germinal vesicle (GV) and metaphase II (MII) stages, as well as in blastocysts derived from both groups, using quantitative real-time PCR. By integrating visual and molecular approaches, this study aims to establish a dual-criterion, field-applicable model for oocyte competence assessment in cattle, with the potential to enhance IVP success rates in practical breeding programs.

Materials and Methods

Collection and Grouping of Oocytes

Ovaries from indigenous zebu cows were collected from a local slaughterhouse within 30–40 minutes of slaughter. The ovaries were transported to the laboratory at the Department of Animal Breeding and Genetics, Bangladesh Agricultural University, in sterile physiological saline (0.9% NaCl) supplemented with 100 IU/mL penicillin and 100 µg/mL streptomycin, maintained at 33–35°C. All oocytes were retrieved within 4–6 hours post-collection. Cumulus-oocyte complexes (COCs) were aspirated from antral follicles ranging from 2 to 12 mm in diameter using an 18-gauge needle attached to a 10-mL syringe. The aspirated follicular fluid was transferred to a sterile petri dish and examined under a stereomicroscope (Amscope SM-2TZZ-LED, UnitedScope LLC, CA). COCs with compact cumulus cell layers and homogeneous ooplasm were selected. Based on visual evaluation of cytoplasmic color under stereomicroscope, the selected oocytes were divided into oocytes with light-colored cytoplasm (LCC) and oocytes with dark-colored cytoplasm (DCC) (Figure 1A).

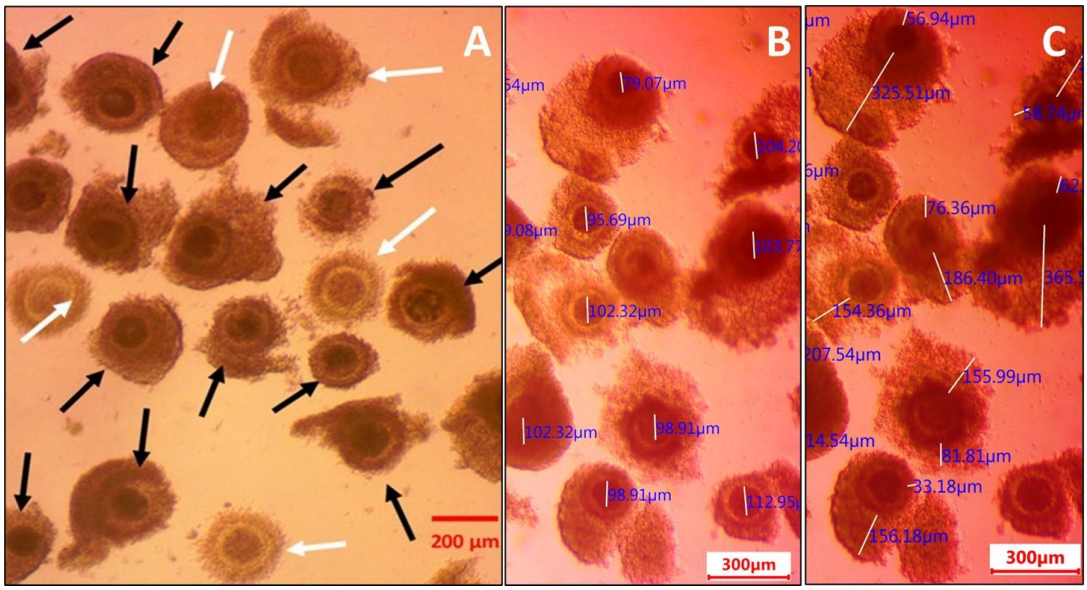

Figure 1. Oocytes categorized by cytoplasmic darkness (A) and measurement of the diameter (µm) of oocyte (B) and length (µm) of cumulus expand (C). White arrow (A) represents oocyte with LCC and black arrow represents oocyte with DCC

In Vitro Maturation (IVM)

IVM was performed in TCM-199 with Earle's salts medium supplemented with 10% estrus cow serum (ECS), 50 µg/mL gentamicin, 0.2 mM sodium pyruvate, 1 mM Ala-Gln, 25 µg/mL folltropin, and 2 µg/mL estradiol. Approximately 20–25 COCs from each group were placed in 100 µL droplets of maturation medium under mineral oil in a four-well dish and incubated at 38.5°C in a humidified atmosphere with 5.5% CO_2 for 24 hours (Figure 3). To assess cytoplasmic maturation, oocyte diameter (Figure 1B) and cumulus expansion (Figure 1C) were measured before maturation at the germinal vesicle (GV) stage and after maturation at the metaphase II (MII) stage. Measurements were taken using image analysis software, and cumulus expansion was expressed as the length (µm) of the outermost cumulus layers.

In Vitro Fertilization (IVF)

Following IVM, matured COCs were washed in modified IVF-TALP medium and co-incubated with motile spermatozoa (2 × 10^6/mL) prepared from frozen-thawed bull semen. Sperm capacitation was achieved by heparin (20 µg/mL) treatment. Fertilization was conducted in 100 µL droplets of IVF medium at 38.5°C in a humidified incubator with 5.5% CO_2 for 18–20 hours.

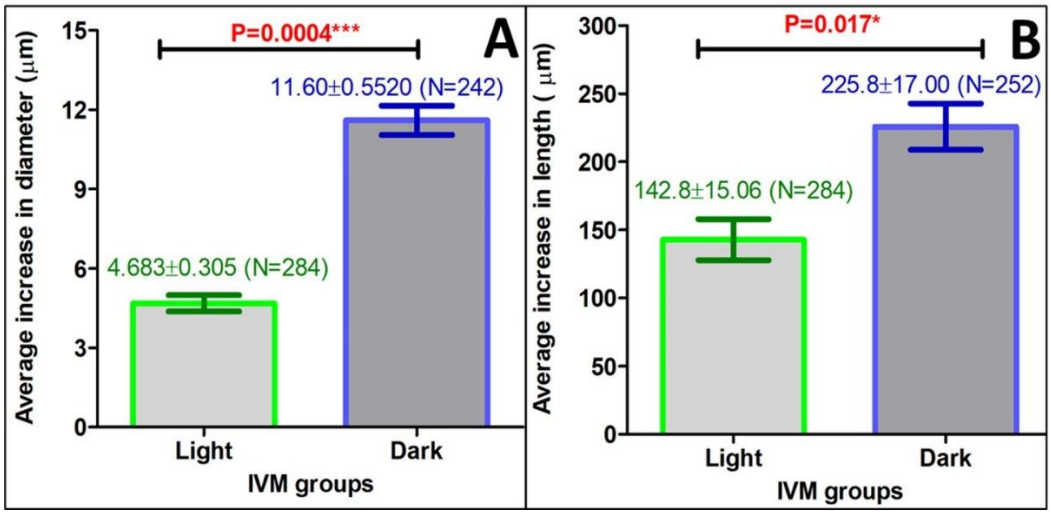

Figure 2. Comparison of average oocyte diameter increase (A) and cumulus expansion (B) after in vitro maturation (IVM) in DCC and LCC groups

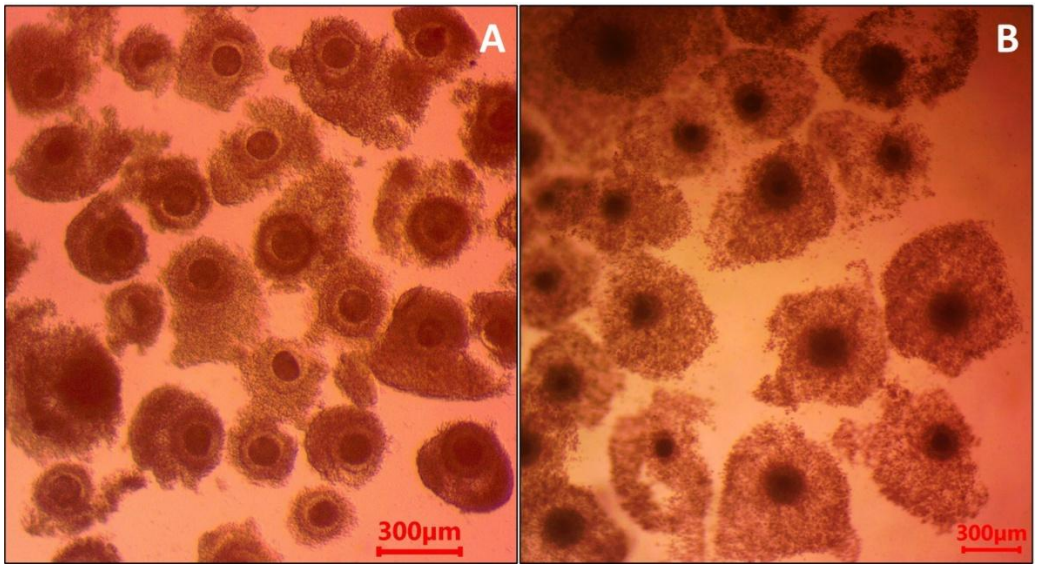

Figure 3. Oocytes at the germinal vesicle (GV) stage (A) and metaphase II (MII) stage (B) within cumulus-oocyte complexes

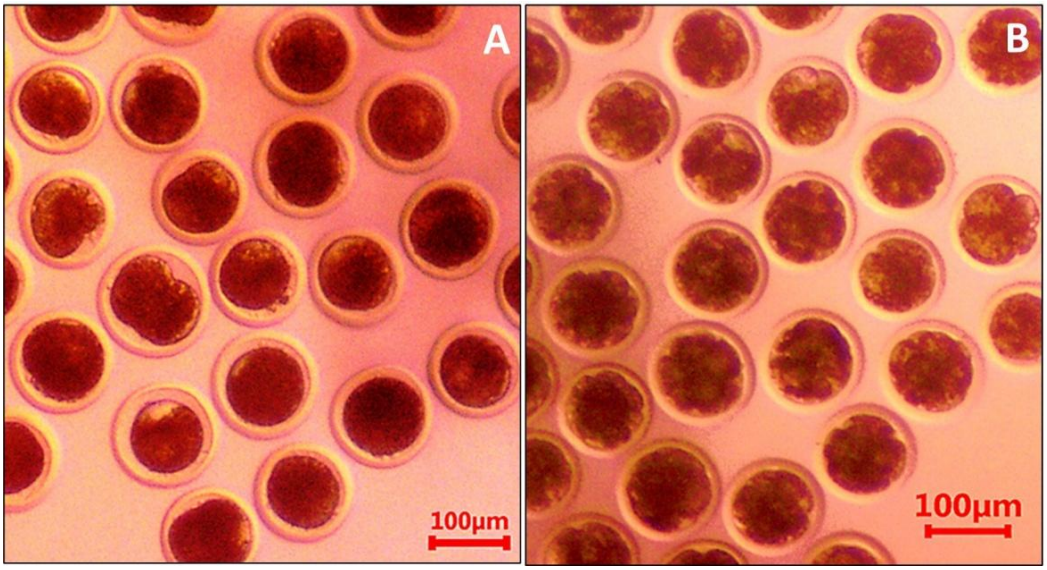

Figure 4. Presumptive zygotes (A) and Day-3 embryos (B)

In Vitro Culture (IVC)

Presumptive zygotes were denuded by gentle pipetting, washed, and cultured in drops of modified SOF-BE2 medium supplemented with amino acids and 4 mg/mL fatty acid-free BSA. Embryos were cultured under mineral oil at 38.5°C in a tri-gas incubator (6% O_2, 5.5% CO_2, 88% N_2) for 8–9 days. Cleavage was assessed on Day 3 and blastocyst development on Day 8 under an inverted microscope (Figure 4).

Quantification of relative expression of PDIA3 transcript

To assess PDIA3 gene expression, total RNA was extracted from GV and MII stage oocytes from DCC and LCC groups using the miRNeasy Mini Kit. Following RNA extraction, 180 ng of RNA from each sample group underwent reverse transcription into cDNA utilizing the AddScript cDNA Synthesis kit (Addbio, Korea) in a thermocycler. A 30 µl reaction mixture underwent incubation at 25°C for 10 minutes, followed by 50°C for 60 minutes, 80°C for 5 minutes, and a final hold at 12°C. Subsequently, 30 µl of the RT reaction mixture was dispensed into each PCR tube. The resultant cDNA was then diluted tenfold, with 4 µl of diluted cDNA utilized per reaction in the subsequent qPCR. Real-time polymerase chain reaction (RT-PCR) was executed employing the PowerUp SYBR Green Master Mix within the MIC qPCR instrument from Biomolecular Systems, Australia. The thermocycler program for qRT PCR encompassed 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute, and a final step of melting curve analysis. All the samples were analyzed in triplicate, and the average value of the triplicate were used for analysis. The data were normalized to GAPDH and 2-ΔΔCt methodology has been used for relative quantification according to the procedure followed previously (Hossain et al., 2009 and 2014).

Statistical Analysis

Data were compiled from a total of 526 COCs (DCC: n=242; LCC: n=284). All results were analyzed using one-way ANOVA followed by Tukey's post hoc test (GraphPad Prism). Statistical significance was considered at $p < 0.05$.

Results

Grouping of Oocytes Based on Cytoplasmic Darkness

In the present study, a total of 526 cumulus-oocyte complexes (COCs) were collected and selected based on morphological quality, specifically the presence of compact cumulus cell layers and homogeneous cytoplasm. These COCs were then classified into two groups based on cytoplasmic appearance as observed under a stereomicroscope. Oocytes with dark or opaque cytoplasm were categorized as dark-colored (DCC, n = 242), while those with transparent or less pigmented cytoplasm were categorized as light-colored (LCC, n = 284) (Figure 1A). The selection was subjective but consistent across all replicates. This grouping allowed for the comparative assessment of oocyte quality and developmental potential.

Oocyte Diameter Before and After Maturation

To evaluate the extent of cytoplasmic maturation during in vitro maturation (IVM), the diameter of oocytes was measured at two distinct time points: before IVM (germinal vesicle stage) and after IVM (metaphase II stage). Initial measurements at the GV stage revealed no statistically significant difference between the DCC and LCC groups in terms of baseline diameter, indicating similar sizes at the beginning of culture (Table 1, Figure 3A). However, after 24 hours of IVM, both groups exhibited an increase in oocyte diameter, but the increase was significantly greater in the DCC group. Specifically, oocytes in the DCC group displayed an average diameter increment of 11.60 ± 0.552 µm, whereas the LCC group showed a mean increase of only 4.683 ± 0.305 µm (Figure 2A). The difference was statistically significant ($p = 0.0004$), suggesting enhanced cytoplasmic maturation in oocytes with darker cytoplasm.

Cumulus Expansion Before and After IVM

Cumulus expansion was assessed as an indicator of nuclear maturation and communication between oocytes and surrounding cumulus cells (Figure 3B). Before IVM, all COCs exhibited tightly packed, multilayered cumulus cells with no visible signs of expansion in either group. Following the 24-hour IVM period, distinct cumulus expansion was observed in both groups, but the extent of expansion differed markedly (Table 2). In the DCC group, the average cumulus expansion length was 225.8 ± 17.00 µm, significantly greater than the 142.8 ± 15.06 µm observed in the LCC group (Figure 2B). This difference was highly significant ($p < 0.001$). The greater expansion in the DCC group suggests that these oocytes had a more robust paracrine interaction with their cumulus cells, likely facilitated by a metabolically active cytoplasm enriched with lipids and mitochondria.

Table 1. One-way ANOVA with Tukey's multiple comparison test on oocyte diameter in two groups before (GV) and after (MII) in vitro maturation

Tukey's Multiple Comparison Test	Mean Diff.	q Value	Significance (p < 0.05)	Summary	95% CI of diff
GV_Light vs GV_Dark	-0.9333	2.973	No	NS	-2.176 to 0.3093
GV_Light vs MII_Light	-4.683	14.92	Yes	***	-5.926 to -3.441
GV_Light vs MII_Dark	-12.53	39.92	Yes	***	-13.78 to -11.29
GV_Dark vs MII_Light	-3.750	11.94	Yes	***	-4.993 to -2.507
GV_Dark vs MII_Dark	-11.60	36.95	Yes	***	-12.84 to -10.36
MII_Light vs MII_Dark	-7.850	25.00	Yes	***	-9.093 to -6.607

Table 2. One-way ANOVA with Tukey's multiple comparison test on cumulus expansion in two groups of COCs before (GV) and after (MII) in vitro maturation

Tukey's Multiple Comparison Test	Mean Diff.	q value	Significance (P < 0.05)	Summary	95% CI of diff
GV Light vs GV_Dark	-8.583	0.8307	No	NS	-49.48 to 32.31
GV Light vs MII_Light	-142.8	13.82	Yes	***	-183.7 to -101.9
GV Light vs MII_Dark	-234.4	22.69	Yes	***	-275.3 to -193.5
GV_Dark vs MII_Light	-134.3	12.99	Yes	***	-175.1 to -93.35
GV_Dark vs MII_Dark	-225.8	21.85	Yes	***	-266.7 to -184.9
MII_Light vs MII_Dark	-91.57	8.862	Yes	***	-132.5 to -50.67

Fertilization Rates Following IVF

The fertilization potential of oocytes from both groups was assessed following co-incubation with capacitated spermatozoa. Fertilization was confirmed by the presence of two pronuclei or early cleavage stages, observed under an inverted microscope 18–20 hours post-insemination. The fertilization rate was significantly higher in the DCC group compared to the LCC group. Specifically, 84.53 ± 2.49% of the oocytes in the DCC group were successfully fertilized, whereas only 67.80 ± 5.37% of the LCC group oocytes showed signs of fertilization, with a statistically significant difference (p = 0.015). This finding indicates that cytoplasmic darkness, presumed to represent higher lipid content, positively influences oocyte fertilization competence.

Cleavage Rates Post-Fertilization

The first mitotic division of fertilized oocytes, termed cleavage, was evaluated on Day 3 post-insemination (Figure 4A). Cleavage is a critical milestone indicating embryonic genome activation and early development. As with fertilization, the cleavage rate was significantly higher in the DCC group compared to the LCC group. In the DCC group, 84.53 ± 2.49% of the fertilized oocytes proceeded to cleavage, while the cleavage rate in the LCC group was 67.80 ± 5.37% (Figure 5A). This difference was statistically significant (p = 0.017). The higher cleavage rate observed in the DCC group reflects greater early developmental potential, which may be linked to a more complete cytoplasmic maturation process that provides essential transcripts, proteins, and organelles for the initiation of mitotic divisions.

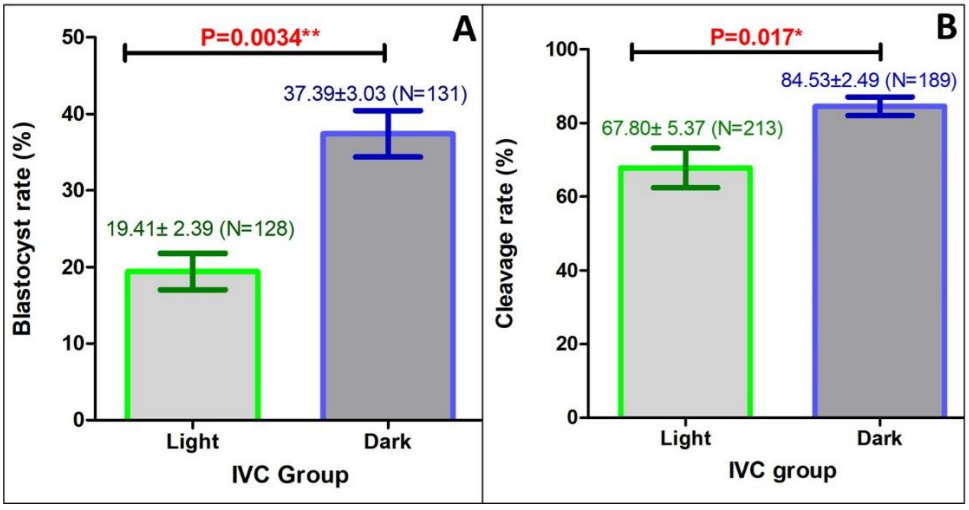

Figure 5. Comparison of cleavage rate (A) and blastocyst formation rate (B) at Day 8 post-IVF in DCC and LCC oocyte groups

Blastocyst Formation on Day 8 of Culture

The final outcome measure of this study was the development of embryos to the blastocyst stage by Day 8 of in vitro culture (Figure 5B and 6). This stage reflects the cumulative effect of oocyte quality, fertilization success, and cleavage competence. The results revealed a highly significant difference between the two groups. In the DCC group, 37.39 ± 3.025% of cleaved embryos progressed to the blastocyst stage, whereas only 19.41 ± 2.391% reached this stage in the LCC group ($p = 0.0034$) (Figure 5B). This represents nearly a twofold increase in blastocyst yield in the DCC group compared to the LCC group. The superior blastocyst formation rate in the DCC group strongly supports the hypothesis that cytoplasmic darkness is a reliable morphological indicator of oocyte developmental competence.

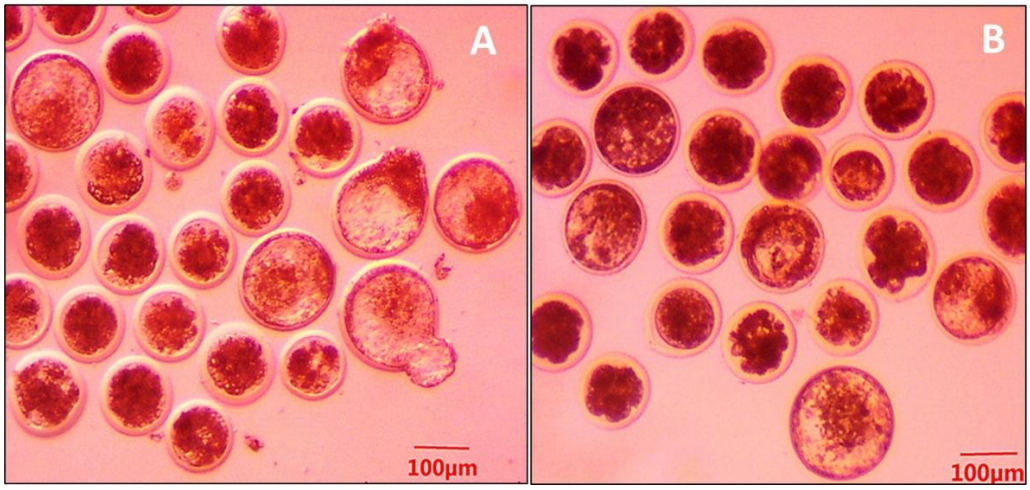

Figure 6. Blastocysts at Day 8 post-IVF from DCC (A) and LCC (B) oocyte groups

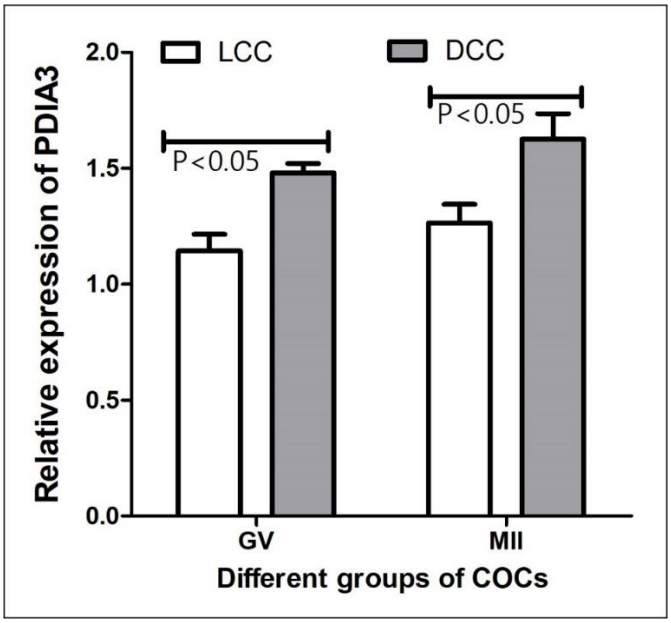

Figure 7. Expression of PDIA3 transcript during maturation in LCLC and HCLC group of cumulus oocyte complex.
Data are expressed as mean ± SEM. LCC: Light-colored cytoplasm, DCC: Dark-colored cytoplasm, Oocyte at germinal vesicle (GV) and metaphase II (MII) stage.

PDIA3 Gene Expression in Oocytes and Blastocysts

Quantitative RT-PCR analysis revealed PDIA3 expression was low and comparable in GV-stage oocytes from both groups. However, in MII-stage oocytes, PDIA3 expression was significantly higher in the DCC group than in LCC ($p < 0.01$) (Figure 7). These findings confirm the upregulation of PDIA3 in morphologically superior oocytes and embryos, consistent with greater cytoplasmic and developmental competence.

Discussion

The present study provides compelling evidence that cytoplasmic coloration, assessed visually under stereomicroscopy, is a reliable and non-invasive indicator of bovine oocyte developmental competence. Oocytes exhibiting dark-colored cytoplasm (DCC) demonstrated significantly superior morphological features during in vitro maturation (IVM), higher fertilization and cleavage rates following in vitro fertilization (IVF), and a markedly greater capacity to develop into blastocysts during in vitro culture (IVC). These findings confirm the hypothesis that cytoplasmic pigmentation reflects the biochemical and metabolic status of the oocyte, and importantly, this morphological assessment was validated at the molecular level through differential expression of the protein disulfide isomerase A3 (PDIA3) gene.

The greater increase in oocyte diameter observed in the DCC group (11.60 ± 0.55 µm) compared to the LCC group (4.68 ± 0.31 µm) strongly indicates more robust cytoplasmic maturation. This enlargement likely reflects a series of coordinated subcellular events including cortical granule migration, mitochondrial redistribution, lipid droplet mobilization, and expansion of the smooth endoplasmic reticulum—all processes essential for oocyte competence (Torner et al., 2004; Stojkovic et al., 2001). These organelle rearrangements contribute to an optimized intracellular environment, enhancing the oocyte's ability to support fertilization and early development.

The significantly greater cumulus expansion in the DCC group (225.8 ± 17.0 µm vs. 142.8 ± 15.1 µm) further supports the hypothesis that DCC oocytes possess higher metabolic readiness. Enhanced expansion in the DCC group thus reflects not only nuclear maturation but also strong biochemical signaling competence, indicating a functional advantage for these oocytes during maturation.

Functionally, DCC oocytes outperformed LCC oocytes across all IVP stages. Fertilization rates in the DCC group reached 84.53 ± 2.49%, significantly higher than the 67.80 ± 5.37% observed in the LCC group. These differences likely arise from superior meiotic spindle formation, mitochondrial ATP production, and cytoskeletal integrity, all of which are dependent on a well-matured and metabolically competent ooplasm (McEvoy et al., 2000; Jeong et al., 2009). Cleavage rates and blastocyst yields followed the same pattern, with DCC-derived embryos displaying significantly higher progression to early developmental milestones, including Day 8 blastocyst formation (37.39 ± 3.03% vs. 19.41 ± 2.39%). These results are consistent with previous studies in pigs and cattle that showed positive correlations between intracellular lipid content and embryo development (Jeong et al., 2009; Lonergan and Fair, 2008).

One of the novel contributions of this study is the molecular validation of the visual oocyte classification through the analysis of an oocyte secreted factor PDIA3 expression. PDIA3 is a multifunctional enzyme involved in disulfide bond formation, protein folding, and oxidative stress regulation in the endoplasmic reticulum (Jingyu Li et al., 2014; Barros et al., 2019). Our results demonstrated significantly higher PDIA3 expression in MII-stage oocytes of DCC group. This observation suggests that PDIA3 plays an active role in oocyte competence, potentially by modulating redox balance and protecting against ER stress during critical transitions such as fertilization and zygotic genome activation.

Importantly, the correlation between visual traits (cytoplasmic darkness) and molecular markers (PDIA3 expression) strengthens the case for integrating morphological and molecular assessments in oocyte selection protocols. While molecular profiling alone may not be practical in field settings, its alignment with simple visual cues provides strong justification for using cytoplasmic color as a proxy for molecular competence. Moreover, this dual-layered approach can significantly enhance embryo production outcomes in resource-limited laboratories that cannot routinely perform high-throughput molecular diagnostics.

Our findings also have broader implications for embryo quality and viability. Embryos derived from DCC oocytes not only showed higher blastocyst formation rates but are also likely to possess improved cryotolerance, mitochondrial function, and post-transfer survival—traits that have been linked to lipid-rich oocytes in prior studies (Stojkovic et al., 2001; Torner et al., 2004). Given the growing demand for scalable and reproducible embryo production protocols in cattle breeding, particularly in developing countries, these results offer practical and cost-effective solutions for improving reproductive efficiency.

Nonetheless, some limitations of the study must be acknowledged. Although PDIA3 served as an effective molecular marker, a broader transcriptomic or proteomic analysis could provide a more comprehensive understanding of the regulatory networks underpinning oocyte competence. Future research should explore the co-expression of PDIA3 with other competence-related genes such as HSP70, BAX, and GDF9, and evaluate downstream impacts on pregnancy establishment and calf outcomes following embryo transfer. Additionally, direct quantification of lipid content through imaging or biochemical assays could further strengthen the physiological basis for cytoplasmic coloration as a competence marker. This study establishes that oocytes with dark-colored cytoplasm exhibit superior morphological, functional, and molecular attributes that collectively enhance in vitro embryo production. The strong association between cytoplasmic darkness and elevated PDIA3 gene expression provides a mechanistic foundation for using visual traits in oocyte selection. Integrating these morphological cues with molecular validation offers a robust, scalable, and practical approach for improving IVP efficiency in cattle, particularly in settings constrained by resources but rich in reproductive potential.

Conclusion

This study provides compelling evidence that cytoplasmic coloration of bovine oocytes, used as an indirect indicator of lipid content, is strongly associated with in vitro developmental competence. Dark-colored oocytes exhibited significantly greater maturation responses, higher fertilization and cleavage rates, and superior blastocyst formation compared to light-colored oocytes. Importantly, these morphological findings were further validated by significantly elevated PDIA3 gene expression in MII-stage oocytes of DCC group, suggesting a molecular basis for the observed developmental superiority. These findings highlight the combined utility of cytoplasmic pigmentation and PDIA3 expression profiling as a practical and reliable tool for selecting high-quality oocytes to improve the success rates of in vitro embryo production in cattle. The integration of morphological and molecular criteria can enhance the precision of oocyte selection in bovine breeding programs, especially in resource-constrained contexts. Future research integrating lipidomic, mitochondrial, and gene expression analyses will help refine this dual-criterion selection strategy and optimize IVP outcomes.

Competing Interest

The authors declare that they have no competing interests.

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