

THE PROLIFERATION OF CRYOPRESERVED BOVINE GRANULOSA CELLS UNDER *IN VITRO* LONG-TERM CULTURE

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ABSTRACT

This study investigated the long-term proliferative potential and senescence-associated characteristics of cryopreserved bovine granulosa cells (cry-bGCs) during extended *in vitro* culture. The cry-bGCs demonstrated a high proliferative capacity, maintaining growth up to passage 20. However, late-passage cells (P15–P20) exhibited signs of replicative senescence, including reduced proliferation rates and increased cell cycle arrest at the G0/G1 phase. Morphometric analyses revealed significant enlargements in both nuclear and cytoplasmic areas, accompanied by the formation of thickened actin filament bundles within the cytoplasm. Furthermore, the decreased nuclear intensity in late-passage cry-bGCs suggested altered chromatin condensation and impaired DNA replication during mitosis. Progressive morphological changes, from homogenous early-passage cells to polymorphic late-passage cells, indicate cytoplasmic expansion and actin remodeling as key senescence markers. These results demonstrate that cry-bGCs possess strong proliferative potential after cryopreservation but gradually undergo replicative senescence during prolonged culture.

Keywords: Cell cycle, cryopreserved bovine granulosa cells, proliferation.

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INTRODUCTION

Growth and development are fundamental and continuous processes in all living organisms, driven by the tightly regulated coordination of cell growth and proliferation (King & Newmark, 2012). In multicellular organisms, cell proliferation ensures tissue formation, regeneration, and functional maintenance, while cell growth supports biosynthetic activities, including expansion of the cytoplasm, organelles, and genomic material (Sharifi et al., 2025; Yu et al., 2025). Together, these processes sustain normal physiological function and developmental progression.

In mammals, the ovary serves as a critical organ for both reproduction and endocrine regulation, hosting dynamic processes such as folliculogenesis, oocyte maturation, and hormone secretion (Alam & Miyano, 2019). Among ovarian cell types, granulosa cells (GCs) play a central role in supporting oocyte development and follicular maturation by producing steroid hormones and paracrine signaling molecules. The proliferative capacity and functional integrity of GCs are vital for normal follicular physiology and female fertility (Wang, 2025). In recent years, bovine granulosa cells (bGCs) have emerged as an important *in vitro* model for studying reproductive endocrinology, ovarian physiology, the stemness, and cell–cell interactions within the follicular microenvironment (Hoang et al., 2019). Beyond their physiological significance, bGCs have been utilized in biotechnological applications such as *in vitro* follicle culture, oocyte maturation systems, and cell-based reproductive studies. Moreover, the long-term preservation of these cells through cryopreservation provides a valuable tool for maintaining genetic resources and facilitating repeated experiments without needing fresh animal material. However, despite maintaining post-thaw viability, cryopreserved bGCs (cry-bGCs) may experience alterations in proliferation dynamics, cytoskeletal organization, and nuclear morphology during prolonged culture.

Continuous *in vitro* subculture induces replicative senescence, a phenomenon

characterized by irreversible cell cycle arrest, enlarged cell and nuclear morphology, and cytoskeletal reorganization (Veronesi et al., 2023). Senescence reflects the cumulative cellular stress of repeated divisions, DNA damage, and telomere shortening (González-Gualda et al., 2021). In cry-bGCs, these features may be further exacerbated by cryo-induced oxidative stress or cryoprotectant toxicity, reducing proliferative potential over successive passages. Therefore, this study evaluated the changes in cell proliferation and morphological characteristics of cry-bGCs to clarify their proliferative capacity under long-term *in vitro* expansion.

MATERIALS AND METHODS

Cell culture

The bovine ovaries were collected from a slaughterhouse and transported to the laboratory within 3 hours. They were washed with 0.9% saline. Single bovine follicles were collected by dissection under a stereomicroscope. The fresh bGCs were collected from the above follicles by aspiration, and the oocyte-cumulus complexes were removed. The cells were cultured in DMEM/Ham's F-12 medium (DMEM-12-A; Capricorn Scientific, Germany) supplemented with 15% fetal bovine serum (FBS-HI-22B; Capricorn Scientific, Germany) and 1% penicillin–streptomycin (15140-122; Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and were passaged at a split ratio of 1:2.

Cell cryopreservation was performed in a cryotube by mixing 1×10^6 cells with 1 ml DMEM/Ham's F-12 medium with 20% FBS, 10% DMSO. Cell suspension was incubated in -20°C for 1 hour and -80°C overnight before long-term maintenance in liquid nitrogen. For cell thawing, the cryotube was incubated at 37°C in a water bath for 1 minute. Cell suspension was mixed with 4 ml culture medium and centrifuged at 1,500 rpm for 5 minutes for cell pellet collection. Cell pellet was re-suspended with culture medium and cultured at 37°C , 5 CO_2 .

Cell density measurement

Cry-bGCs were seeded at a density of 5×10^2 cells per well in 96-well plates (161093,

Thermo Fisher Scientific, Waltham, MA, USA). After 3 days, cells were fixed and nuclei were stained with Hoechst 33342 (14533, Sigma-Aldrich, Munich, Germany) for 15 minutes, followed by three washes with phosphate-buffered saline (PBS; Gibco, Thermo Fisher Scientific, Waltham, MA, USA). The total cell number was quantified using the Cell Cycle App. of Cytell Cell Imaging System (GE Healthcare, USA).

Microfilament staining and nuclear morphology evaluation

Cry-bGCs were fixed with 4% paraformaldehyde (Nacalai Tesque, Kyoto, Japan) for 30 minutes and subsequently permeabilized overnight with 0.1% Triton X-100 (Merck, Darmstadt, Germany) at 4 °C. Actin filaments were stained with phalloidin CruzFluor™ 488 (sc-363791; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 hour, and nuclei were counterstained with Hoechst 33342 for 15 minutes. After three washes with phosphate-buffered saline, samples were imaged using a Cytell Cell

Imaging System. The Cell Cycle App quantified nuclear area, nuclear fluorescence intensity, nuclear shape, and cell cycle progression provided by the Cytell system (Ho et al., 2021).

Statistical analysis

The experiments conducted in this study were repeated at least three times. Data are presented as mean $\pm$ standard deviation (SD). Statistical analyses were performed using one-way analysis of variance (ANOVA), and differences were considered statistically significant at $p < 0.05$.

RESULTS

The cry-bGCs from P5 showed high proliferation, demonstrated by a high density at 3.57×10^3 cells/well. Compared to cry-bGCs from P5, cry-bGCs from P10 (2.44×10^3 cells/well) and P15 (1.2×10^3 cells/well) showed reduced proliferation. The cry-bGCs from P20 exhibited a marked decrease in *in vitro* proliferation. These results indicated a decreased proliferation of cry-bGCs during long-term *in vitro* expansion.

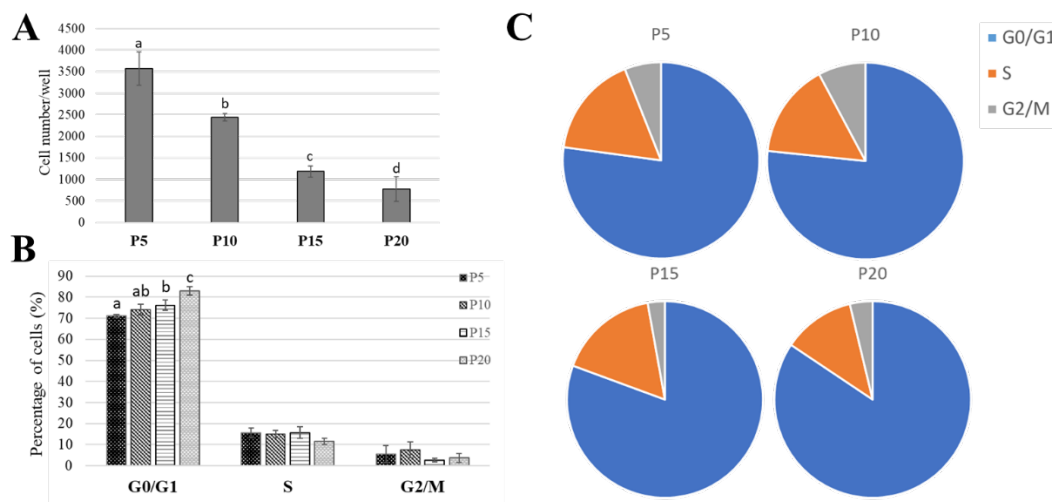


Figure 1. Cell proliferation analysis. A: cell density, B, C: cell cycle progression analysis. a, b, c, d: statistically significant difference ($P < 0.05$)

The cell cycle analysis demonstrated that the lowest percentage in the G0/G1 phase was determined in cry-bGCs from P5 (71.14 ± 0.74 %). The G0/G1 cell ratio was increased

in P10 (74.11 ± 2.62 %) and P15 (76.19 ± 2.39 %); however, there is no difference in the G0/G1 ratio of cry-bGCs between the two passages. The highest G0/G1 ratio was

observed in cry-bGCs from P20 ($82.92 \pm 1.97\%$). These results revealed an increase in the G0/G1 ratio in cry-bGCs during long-term *in vitro* expansion.

The present study assessed the changes in morphological characteristics of cry-bGCs

during long-term *in vitro* culture. As seen in Figure 2A, there was no difference in nuclear area of cry-bGCs from P5 ($159.70 \pm 4.29 \mu\text{m}^2$), P10 ($178.00 \pm 6.01 \mu\text{m}^2$), and P15 ($166.84 \pm 7.77 \mu\text{m}^2$). The cry-bGCs from P20 showed an increase in nuclear area ($199.83 \pm 17.14 \mu\text{m}^2$).

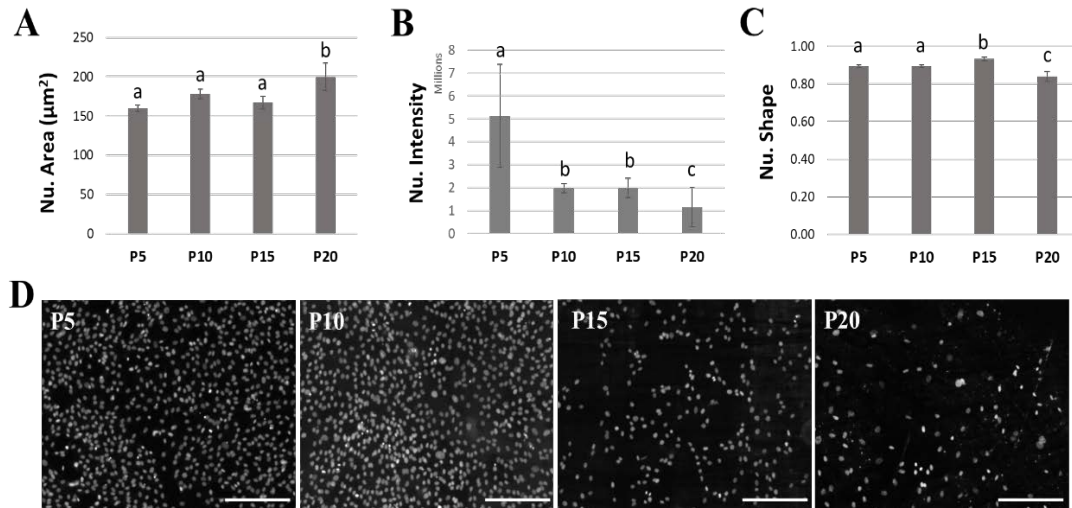


Figure 2. Morphology analysis of cry-bGCs. A: Nuclear area, B: Nuclear intensity, C: Nuclear shape. D: Representative images of nuclei stained with Hoechst 33342. Original magnification $\times 100$. Scale bar = $223.64 \mu\text{m}$. a, b, c: statistically significant difference ($P < 0.05$)

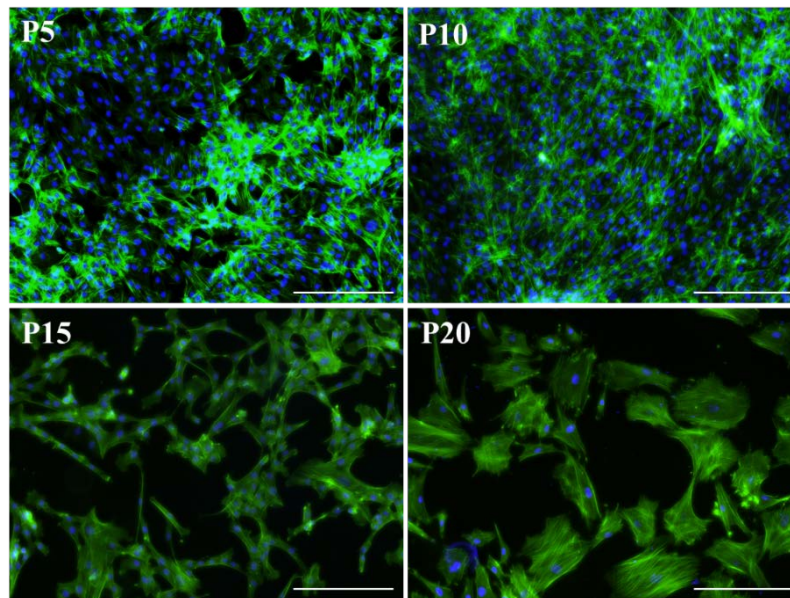


Figure 3. Morphological changes of cry-bGCs during *in vitro* expansion. Actin filament bundles were stained with Phalloidin CruzFluor™ 488 Conjugate (green), and nuclei were stained with Hoechst 33342 (blue). Original magnification $\times 100$. Scale bar = $223.64 \mu\text{m}$

The cry-bGCs exhibited the highest nuclear intensity (5.1×10^6) in P5 (Fig. 2B). The nuclear intensity was reduced in cry-bGCs from P10 (1.9×10^6) and P20 (1.9×10^6). The cry-bGCs from P20 showed the lowest nuclear intensity (1.0×10^6). The nuclear shape value was also applied to assess the cell morphology. Figure 2C, 2D show that cry-bGCs displayed a decreased nuclear shape value from P5 (0.89 ± 0.01) to P20 (0.84 ± 0.03).

Figure 3 shows the microfilament bundles distribution of cry-bGCs during the *in vitro* expansion. In P5 and P10, there were a few microfilament bundles distributed throughout the cytoplasm of the cry-bGCs. The cry-bGCs from P15 and P20 had a dramatically higher number of microfilament bundles, which correlated to the cytoplasm expansion.

DISCUSSION

This study found that the cry-bGCs showed a high potential in the long-term *in vitro* proliferation, which was indicated by the capacity of cry-bGCs in reaching passage 20. However, the cry-bGCs in late passages exhibited several senescence characteristics, as demonstrated by the reduced proliferation and the elevated cell cycle arrest in G0/G1 in passage 15 and passage 20. Moreover, the senescent properties were observed in these, such as the expansion of nuclear area, cell area, and an increase of microfilament bundles in the cytoplasm of cry-bGCs. These results suggested that cry-bGCs underwent replicative senescence during long-term *in vitro* expansion.

The size of the nucleus may play a crucial role in accommodating the nucleolus, enzymes like DNA polymerase, and macromolecules (Li et al., 2024; Efremov et al., 2022). According to the earlier research, nuclear size is influenced by one or more cellular factors. Nuclear size is governed by cytoplasmic volume rather than DNA content, even though genomic size is a significant nuclear component but does not influence nuclear size (Mukherjee et al., 2016). The present investigation showed a significant increase in the nuclear area of cry-bGCs, indicating that these cry-bGCs displayed an

amplification of transcription necessary in larger cells.

Nuclear reconstruction is required for cell division since it speeds up the creation of other cellular components and DNA replication. Mitosis requires the process of chromatin condensation, which raises nuclear intensity (Habela & Sontheimer, 2007). According to this study, cry-bGCs from late passages had a lower nuclear intensity than those from early passages, which may indicate that these late passaging circumstances catalyzed the alterations in nuclear reconstruction and/or DNA replication.

Replicative senescence caused cry-bGCs to undergo phenotypic alterations, such as nucleus, cell expansion, and the appearance of massive actin filament bundles in the cytoplasm (Casorati et al., 2025). In the early passages, the cry-bGCs kept their homogenous morphology. The cry-bGC populations from late passages developed polymorphotypes due to cytoplasmic expansion. A strong expansion and variable cytoplasmic localization were seen in the cry-bGCs from passage 20, which were larger and more morphotypic than the cry-bGCs from early passages. Consequently, throughout *in vitro* growth, cry-bGCs sustained a progressive morphological change under long-term *in vitro* culture, and for replicative senescence, this biomarker is crucial.

The morphological changes observed in cry-bGCs are primarily driven by remodeling of the actin cytoskeleton. After cryopreservation, mechanical stress and membrane tension shifts alter the formation of microfilament bundles, which progressively increase during extended culture (Müllers et al., 2019). These reinforced fibers elevate intracellular contractility, producing the characteristic flattened and enlarged cell morphology. As cells spread more widely and develop greater cytoskeletal stiffness, they generate stronger traction forces that stabilize this altered phenotype (Chinnadurai et al., 2014). The enhanced actin tension also reshapes nuclear shape. The actin cytoskeleton remodeling serves as the key mechanistic link between cryopreservation-induced stress and persistent alterations in both nuclear morphology and cell morphology.

CONCLUSION

In conclusion, cry-bGCs showed strong proliferative ability up to passage 20. However, long-term culture induced replicative senescence in later passages. Senescence was marked by reduced proliferation and G0/G1 arrest. Cells exhibited enlarged nuclei, expanded cytoplasm, and dense actin bundles. Decreased nuclear intensity suggested impaired chromatin condensation. These findings highlight progressive senescence and morphological remodeling in cry-bGCs during extended *in vitro* culture.

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