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SIMULATED MICROGRAVITY ALTERED THE CELL CYCLE PROGRESSION OF PORCINE GRANULOSA CELLS

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SUMMARY

Microgravity has been shown to markedly affect reproduction in humans and animals, especially reproductive organs such as the ovaries. Granulosa cells are one of the important components of the ovary, playing an important role in supporting oocyte maturation and fertilization. However, the effects of microgravity on granulosa cells have not been well characterized. This study aimed to assess the effects of simulated microgravity (SMG) on the cell cycle progression of porcine granulosa cells (pGCs). The pGCs were induced SMG for 72 h by Gravite® simulator, while cells of the control group were treated in normal conditions. Cell cycle analysis revealed that SMG condition induced an increase of the ratio of pGCs in the G0/G1 phase, leading to the cell cycle arrest phase, while the ratio of pGCs in the G2/M phase was decreased. There was no difference in the cell ratio of the S phase between the control group and the SMG group. Real-time RT-PCR analysis indicated that the expression of *cdk4* and *cdk6* transcripts of pGCs from the SMG group was lower than the control group. This down-regulation was also observed *cyclin A* and *cyclin D1* transcript expression in pGCs from the SMG group. Immunostaining displayed the lower exhibition of microfilament and microtubule in pGCs from the SMG group comparing to the control group. Western blot analysis showed that the expression of β-actin and α-tubulin was reduced in pGCs from the SMG group. These changes contributed to the alteration of cytoskeletal structure, including microfilaments and microtubules, which affect cell division. These results revealed that the SMG condition induced changes in the cell cycle progression of pGCs.

Keywords: cell cycle, cytoskeletal proteins, granulosa cells, proliferation.

INTRODUCTION

The abnormalities in fertilization and embryonic development in sea urchins and amphibians were reported to be caused by microgravity from space flight (Souza *et al*., 1995; Schatten *et al*., 1999; Aimar *et al*., 2000). Another study showed that the mating, fertilization, and hatching processes of Medaka fish during orbit could give the next offspring with normal ovaries and fertility (Ijiri, 1998). Space flights also decrease testicular weight, reduced testosterone hormone levels, and androgen receptor expression in the testes (Strollo *et al*., 1998; Kamiya *et al*., 2003). SMG also induced the abnormality in the germinal epithelium and resulted in the degeneration and necrosis in the seminiferous tubules by an up-regulation of Hsp70 and the activation of the apoptotic pathway in experimental animal

models (Sharma *et al*., 2008).

The ovary is a highly organized structure containing eggs and other somatic cells, including granulosa cells, thecal cells, and stromal cells. These cells play an important role in oocyte development, ovulation, and the formation of the corpus luteum (Richards and Pangas, 2010). The previous study demonstrated that oocytes and their surrounding ovarian somatic cells are highly sensitive to destruction by high charge and energy particles typical of space radiation (Mishra, Luderer, 2019). SMG inhibited GDF-9 expression in oocytes compared to the control group (Zhang *et al*., 2016). The spindle organization and cytoplasmic blebbing were observed in GV and MI oocytes under SMG conditions (Wu *et al.*, 2011). In addition, SMG induces apoptosis in endothelial cells (Maier *et al.*, 2015). However, the effects of SMG on the morphology and proliferation of granulosa cells have not been well characterized. The objective of the present study was to estimate the in vitro effects of SMG on the cell cycle progression of pGCs which play an important role in cell proliferation.

MATERIALS AND METHODS

pGC isolation

The ovaries were collected from the abattoir. The dissection method was applied to collect follicles under a stereo-microscope (Meiji, Japan) (Son *et al.* 2019). The pGCs were isolated from the single follicles by aspiration. The oocyte-cumulus complexes were removed. The pGCs were cultured DMEM/Ham's F-12 (DMEM-12-A, Capricorn Scientific, Germany), with 15% FBS (FBS-HI-22B, Capricorn Scientific, Germany) and 1% Pen/Strep (PS-B, Capricorn Scientific, Germany).

Microgravity simulation

pGCs were seeded in 96-well plates at a density of 1×10^3 cells/well. Each well was carefully filled with the culture medium (avoiding bubbles formation) (Leguy *et al.* 2011). pGCs were induced SMG condition for 72 h by Gravity Controller Gravite® (AS ONE INTERNATIONAL, INC., Santa Clara, CA, United States). The Gravite® was placed in CO_2 incubator (MCO-18AIC, Sanyo Electric Co., Japan) (Figure 1C). The pGCs from control group were cultured in the same CO_2 incubator at 1G.

Cell cycle analysis

pGCs were incubated with 4% paraformaldehyde in PBS (Nacalai, Japan) for 30 min. 0.1% Triton X-100 in PBS was used to permeabilize pGCs. The nucleus was stained with Hoechst 33342 (14533, Sigma-Aldrich, United States) for 30 min. pGCs were washed with PBS three times in 5 min for each step. Cell cycle progression was determined by Cell Cycle App. of Cytell microscope (GE Healthcare, United States).

Quantitative real-time RT-PCR

Total RNA extraction was conducted by a ReliaPrepTM RNA Cell Miniprep System (Z6011, Promega, USA). The reaction was carried out in 20 µL reaction including 1 µL of total RNA, 1 µL of primers (forward and reverse), 10 µL 2X Mix Hi-ROX (PCRBiosystem, England), 1 µL RTAse, and 7 µL dH_2O. The qRT-PCR reaction was performed by one cycle of 45°C for 15 min, one cycle of 95°C for 2 min, 40 cycles of 95°C for 10 sec, 60°C for 15 sec and 71 cycles of 60°C for 15 sec. Primers were as follows: *cdk4*, F 5'-TTC GAG CAT CCC AAT GTT GTC -3' and R 5'-GTC TCG ATG AAC GAT GCA GTT G -3'; *cdk6*, F 5'-TTG GCT TTG GTG GGT AGT TCT-3' and R 5'-TGA ATG TCG GGT AGG AAG ATT G-3'; *cyclin D1*, F 5'-TGC ATC TAC ACC GAC AAC TCC A -3' and R 5'-GTT GGA AAT GAA CTT CAC GTC TGT-3'; *gapdh*, F 5'-ATG GTG AAG GTC GGA GTG AAC-3' and R 5'-CTC GCT CCT GGA AGA TGG T-3' (Yang *et al*., 2021); *cyclin A*, F 5'-GAT TTA CAT CTT AGA AAA CAA AGG-3' and R 5'-GGT GAT CCC GCC GTC CAC T-3' (Tang *et al.*, 2010). The $2^{-\Delta\Delta Ct}$ method was applied for Ct value analysis (Livak, Schmittgen, 2001).

Western blot

pGCs were treated with Optiblot LDS Sample Buffer (ab119196, Abcam, United States). The protein samples were loaded into the wells of the Precast Gel SDS-PAGE 4-12 % (ab139596, Abcam, United States) with an equal amount. The gel was run in Optiblot SDS Run Buffer (ab119197, Abcam, United States) for 2 h at 50 V. The protein samples were transferred to methanol-treated PVDF membrane (ab133411, Abcam, United States), then the membrane blocking was carried out overnight at 4°C with Blocking buffer (ab126587, Abcam, United States). The membrane was incubated with a primary antibody overnight at 4°C. Anti-beta Actin antibody (ab8226, Abcam, United States) and Anti-alpha Tubulin antibody (ab52866, Abcam, United States) were used at 1:10000 dilution. Anti-GAPDH antibody (ab181602, Abcam, United States) was used as the control at 1:10000 dilution. The Goat Anti-Mouse IgG (HRP) (ab6789, Abcam, United States) and the Goat Anti-Rabbit IgG (HRP) (ab6721, Abcam, United States) were used against beta Actin antibody and other primary antibodies, respectively. The blot visualization was performed by ECL Kit (ab65623, Abcam, United States) with X-Ray film.

Microfilament staining

pGCs were treated with 4% paraformaldehyde in PBS (Nacalai, Japan) for 30 min, then cells were permeabilized with 0.1% Triton X-100 in PBS (Merck, Germany) for 30 min. Phalloidin CruzFluor™ 488 Conjugate (Santa Cruz Biotechnology, United States) was applied to actin filament staining. The nuclear was stained with Hoechst 33342 (14533, Sigma-Aldrich, Germany) for 30 min. The pGCs were washed with PBS three times in 10 min between each step. The pGC microfilament distribution was observed under Cytell microscope.

Microtubule staining

Microtubules of pGCs were stained with 50 nM SiR-tubulin/well (CY-SC002, Cytoskeleton, Inc., United States). pGCs were induced SMG for 72 h. pGC nuclear were counterstained with Hoechst 33342 for 30 min. The microtubule bundle distribution was estimated under a Cytell microscope.

Statistical analysis

The data were analyzed for statistical significance by one-way ANOVA, where $P<0.05$ was considered statistically significant.

RESULTS

The pGCs from the control group showed a morphology in fibroblast-like appearance, including being bipolar or multipolar, and elongated shapes. pGCs from the control group also displayed higher density and expansion than SMG conditions (Figure 1A). This result revealed that SMG induced a reduced proliferation in pGCs. The present work estimated the cell cycle progression by nuclear staining and analyzed by Cell Cycle App. of Cytell Microscope (Figure 1). The ratio of SMG-induced pGCs in the G0/G1 phase was higher than the control cells (58.62 ± 1.97 % vs. 47.53 ± 2.22 %, respectively) ($P < 0.001$). There was no difference in the cell ratio of the S phase in pGCs between the control group and the SMG group (15.07 ± 4.04 % vs. 15.01 ± 2.45 %). pGCs from the SMG condition showed the lower the ratio in the G2/M phase compared to the control group (23.05 ± 2.54 % vs. 34.96 ± 2.09 %) ($P < 0.001$) (Figure 1B). These results demonstrated that the SMG condition enhanced the transition of pGCs to the arrest phase.

The transcript expression of cell cycle-related genes was evaluated by real-time RT-PCR. The expression of *cdk4* transcript in pGCs from the SMG group was lower than the control group ($P = 0.03$). The down-regulation was also observed in *cdk6* transcript expression in pGCs under SMG condition ($P = 0.023$) (Figure 2). These results indicated that SMG induced a reduction of the expression of the main cdk transcript in pGCs.

Moreover, the cyclin transcript expression of pGCs under SMG conditions was also assessed.

pGCs from the SMG group exhibited a decreased expression of *cyclin A* transcript, comparing to the control group ($P < 0.001$). However, there was no difference in *cyclin D1* transcript expression between the control group and the SMG group (Figure 3).

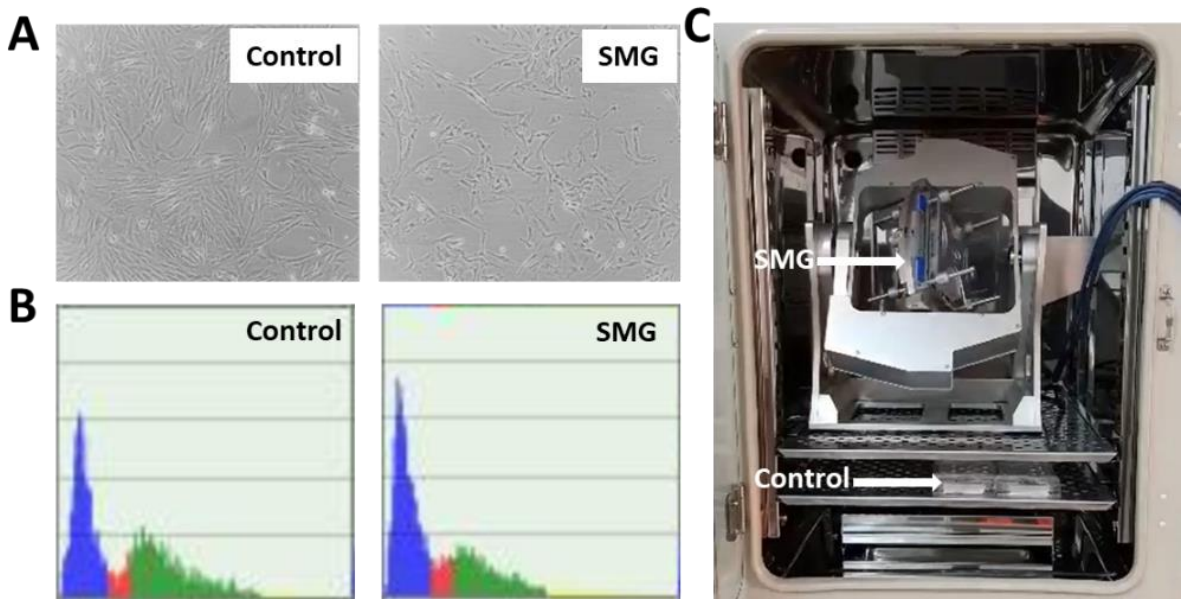

Figure 1. Cell proliferation. A. Morphology of pGCs from the control group and the SMG condition. B. Cell cycle analysis by Cell cycle App. of Cytel microscope, the blue indicates the G0/G1 phase, the red indicates the S phase, and the green indicates the G2/M phase. C. Gravite operation in a CO_2 incubator.

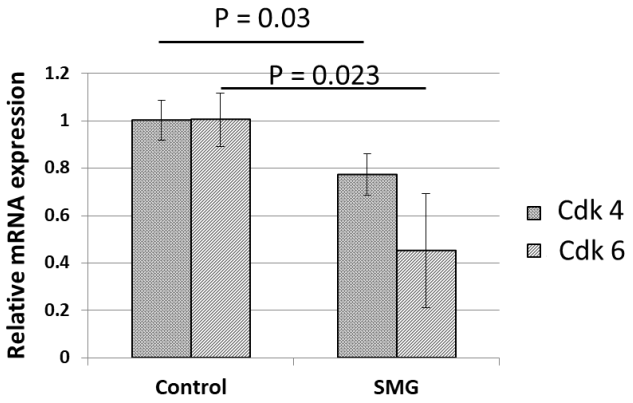

Figure 2. The transcript expression analysis of *cdk4* and *cdk6* by real-time RT-PCR. The pGCs from the SMG group showed that there was a down-regulation of *cdk4* and *cdk6* compared to the control group.

In this study, we found that the SMG condition induced changes in the expression of major cytoskeletal proteins. The immunostaining analysis demonstrated that the exhibition of microfilament and microtubule in pGCs from the SMG group was lower than the control group (Figure 4A). The western blow analysis showed that the β-actin expression in pGCs of the SMG group was lower than the control group (Figure 4B). As shown in Fig. 4B and 4D, the pGCs from the SMG group exposed a slight decrease of α-tubulin expression, compared to cells from the control group.

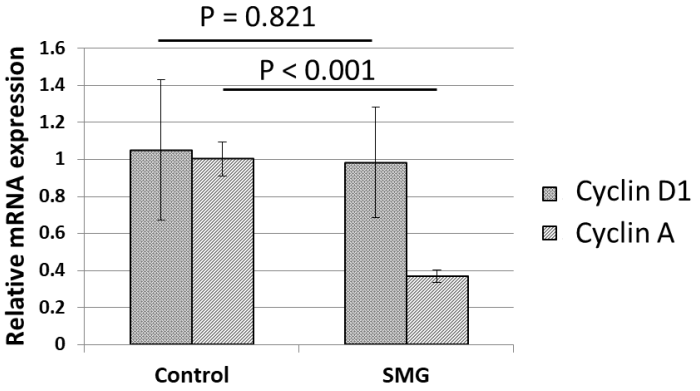

Figure 3. The transcript expression analysis of *cyclin A* and *cyclin D1* by real-time RT-PCR. The pGCs from the SMG group showed the down-regulation of *cyclin A* and *cyclin D1* compared to the control group.

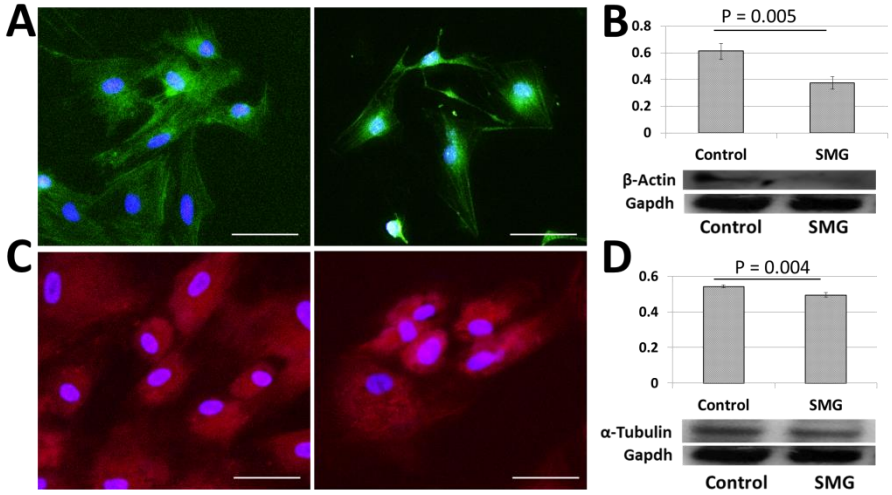

Figure 4. The expression of main cytoskeletal proteins. A, C: Microfilament and microtubule staining. The microfilaments were counterstained with Phalloidin (green), microfilaments were counterstained with Sir-Tubulin (red), and nuclei were counterstained with H33342 (blue). B, D: The expression analysis of β-actin and α-tubulin by Western blot.

DISCUSSION

Space travel markedly affected metabolism, gene regulation, microbiome composition, and immune system in humans (Garrett-Bakelman *et al.*, 2019), especially in human reproduction. Reproductive changes could arise at the hypothalamus or pituitary. Ovulation failure can be caused by stress. The stress endured during spaceflight may be linked to detrimental effects on the ovulatory cycle. Spaceflights expose humans to a variety of reproductive risks, including radiation, microgravity, and hypergravity (Mishra, Luderer, 2019). In the ovary, granulosa cells are crucial for follicle development by supporting the oocyte growth and maturation, producing sex steroids and growth factors (Tu *et al.*, 2019). The changes in function and structure of granulosa cells could affect ovary physiology. In the present work, SMG condition induced attenuation in pGC proliferation, by demonstrating the changes in cell cycle progression. The percentage of pGCs was increased in the G0/G1 phase and decreased

in the G2/M phase, suggesting that the arrest phase transition resulted in the decreased proliferation of SMG-induced pGCs.

The cell cycle progression is controlled by cell cycle-related genes. Cdk4 and cdk6 associate with cyclinD to form a complex which promotes the transition from the G1 phase to the S phase (Tigan *et al.*, 2016). This transition plays a key role in controlling cell proliferation (Tigan *et al.*, 2016). The inhibition of cdk4/6 causes the reduction of cell proliferation (Kollmann *et al.*, 2019; Bonelli *et al.*, 2019). This study found that the expression of *cdk4*, *cdk6*, and *cyclin D1* transcripts was decreased in pGCs under SMG conditions. In addition, the down-regulation of *cyclin A* transcript was observed in SMG-treated pGCs. These resulted in the delay in the G1/S phase transition of pGCS under SMG conditions, leading to the arrest phase.

The effects of microgravity on female reproduction have been linked to increased oxidative stress which induces the changes in cell proliferation and morphology of ovarian cells. Changes in cellular morphology caused by microgravity indicate changes in cytoskeletal structures such as microtubules and actin filaments (Thiel *et al*., 2019; Prasad *et al*., 2020). The cytoskeleton plays a crucial role in maintaining cellular organization, division, and movement. The polymerization of tubulin forms microtubules. Microtubules contribute to spindle formation that separates the chromatids into sister cells (Cohen-Fix, 2000). Actin is a component of microfilaments that supports the formation of the cleavage furrow during cell division (Spira *et al.*, 2017). In this investigation, SMG condition induced the alteration of the main cytoskeletal proteins in pGCs. In this study, we found that the SMG condition induced the down-regulation of α-actin and β-tubulin in pGCs. This has as a consequence of alteration in cytoskeleton structure and division of pGCs treated by SMG condition.

CONCLUSION

This study found SMG condition gives rise to the transcript down-regulation of the main cell cycle-related genes and the attenuation of major cytoskeletal proteins. These alterations induced changes in the cell cycle progression of pGCs under SMG conditions.

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