

INHIBITED GROWTH OF MESENCHYMAL STEM CELLS UNDER SIMULATED MICROGRAVITY

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Received: 07.5.2020

Accepted: 28.6.2020

SUMMARY

This study aimed to estimate the effects of simulated microgravity (SMG) on mesenchymal stem cells (MSCs). The 3D clinostat was applied to induce simulated microgravity on MSC. The results showed that the MSC density in control group was higher than the SMG group. The cell cycle analysis demonstrated that the MSC ratio in G0/G1 phase in SMG group was higher than that of the control group, while the MSC ratio in S phase and G2/M phase in SMG group was lower than those of the control group. The real time quantitative RT-PCR was used to evaluate the expression of cell cycle-related genes, including Cyclin-dependent kinase 2 (Cdk2), Cyclin-dependent kinase 6 (Cdk6), and Cyclin A. The results showed that the transcript expression of Cdk2, Cdk6, and Cyclin A was down-regulated in MSC from SMG group comparing to that of the control group. The flow cytometry was performed to determine the ratio of apoptotic MSCs. There was no significant difference in viability ratio and apoptotic ratio of MSCs between SMG group and control group. The MSCs from SMG group and control group showed similar in Bcl2 and Bax transcript expression.

Keywords: 3D clinostat, apoptosis, cell cycle, mesenchymal stem cells, simulated microgravity

INTRODUCTION

Gravity is a constant which always affect both physical and biological phenomena during the development of life on Earth. Gravity plays a key role in the evolution of the shape of the organism (Häder et al., 2005). To counteract gravity, the living organism needs to develop supportive systems such as the proper structure, the cell membrane becoming rigid, and regulating the flow of fluid in the body (Bizzarri et al., 2015). The effects of gravity on biological research can now be done through space flight or ground-based simulators. Several models of simulated microgravity systems have been established on the ground such as 2D clinostat, 3D clinostat, RPM (random position machine), rotating chamber, and falling tower to study the impact of zero gravity on the ground (Kim et al.,

2017b; Wuest et al., 2015; Barrila et al., 2018; Wang et al., 2017; Srinivasan et al., 2018). One of the most biological models that has been used to evaluate the effects of simulated microgravity is mesenchymal stem cells. The changes of MSCs have been characterized under SMG inducing with 2D-clinostat (Cazzaniga et al., 2016). The 2D-clinostat has been demonstrated from the first introduction of 1879 (von Sachs, 1879). Even though this model induces the simulated microgravity, it also accompanies to undesirable effects (van Loon, 2007). For this reason, the 3D-clinostat has been developed to produce more closely simulated microgravity (Herranz et al., 2013; Brungs et al., 2016). However, the modifications of MSC in 3D clinostat simulated have not been clearly demonstrated. The present study applied 3D clinostat to assess the modification of MSCs

under simulated microgravity conditions.

MATERIALS AND METHODS

MSCs culture

MSCs were cultured in DMEM/Ham's F-12 (Capricorn Scientific, Germany) supplemented with 15% FBS (Capricorn Scientific, Germany) and 1% Pen/Strep (Gibco, United States) at 37°C in a 5% CO₂ atmosphere. The cells were exposed to fresh culture medium every two days.

Cell density evaluation

MSCs were seeded on 96-well culture plate for the density of 2×10^3 cells/well. MSCs were filled up with culture medium and induced SMG by 3D clinostat system for 72 hours (Figure 1D).

WST-1 kit was applied to determine cell density. Each well was added by 10 µl of WST-1 and incubated for 3.5 h. The O.D. value was measured at 450 nm using Microplate Reader.

Cell cycle analysis

MSCs were cultured in 96-well plate for the density of 2×10^3 cells/well. MSCs were induced SMG by 3D clinostat system for 72 hours. 4% paraformaldehyde (Nacalai, Japan) was used to fix MSCs for 30 min, then cells were permeabilized with 0.1% Triton X-100 (Merck, Germany). MSC nucleus was stained with Hoechst 33342 (Sigma, United states) for 30 min. Cells were washed three times with PBS (Capricorn Scientific, Germany). MSCs were observed under the fluorescent microscope and cell cycle was determined by Cycle App of Cytell Microscope.

Quantitative real time-PCR analysis

The MSCs were harvested and washed with PBS. Total RNA was isolated using a Ribospin Total RNA Purification Kit (GenAll Biotechnology Co., Korea). The quality and quantity of the RNA sample were assessed using the NanoVue Plus spectrophotometer. The PCR reaction included: 1 µl of total RNA, 2 µl of primers, 10 µl Mix Ro-Lox, 1

µl RTase, in a total volume of 20 µl in each reaction. The thermal cycle was performed as follow: 1 cycle at 45°C for 15 min; initial denaturation at 95°C for 2 min; 40 cycles at 95°C for 10 s, 62°C for 15 s; and melting curve analysis was carried out from 60°C to 95°C with the rising of 0.5°C every 15 s. Primers were as follows: CDK2-F: 5'-CAG GAG TTA CTT CTA TGC CTG A-3' and CDK2-R: 5'-TTC ATC CAG GGG AGG TAC AAC-3'; CDK6-F: TCT TCA TTC ACA CCG AGT AGT GC-3' and CDK6-R: TGA GGT TAG AGC CAT CTG GAA A-3' (Ren *et al.*, 2016); Cyclin A-F: 5'-GCC ATT AGT TTA CCT GGA CCC AGA-3' and Cyclin A-R: 5'-CAC TGA CAT GGA AGA CAG GAA CCT-3' (Tessari *et al.*, 2003), GAPDH-F: 5'-CAT GAG AAG TAT GAC AAC AGC CT-3' and GAPDH-R: 5'-AGT CCT TTC CAC GAT ACC AAA GT-3' (Gorgogietas *et al.*, 2018). The comparative Ct method was applied for quantitative data analysis (Livak, Schmittgen, 2001).

Flow cytometry

The FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, United States) was used for apoptosis analysis. The MSCs were incubated with 100 µL Binding Buffer added 5 µL of FITC Annexin V and 5 µL PI for 15 min at room temperature. MSCs were resuspended with 400 µL Binding Buffer and analyzed by BD Accuri C6 Plus.

Statistical analysis

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

RESULTS AND DISCUSSION

The density of 2×10^3 cells/well was applied for MSC seeding. After 3 days of culture, the MSC density of SMG group was 3.06×10^3 cells/well which was lower than that of the control group (3.77×10^3 cells/well) ($P < 0.001$) (Figure 1).

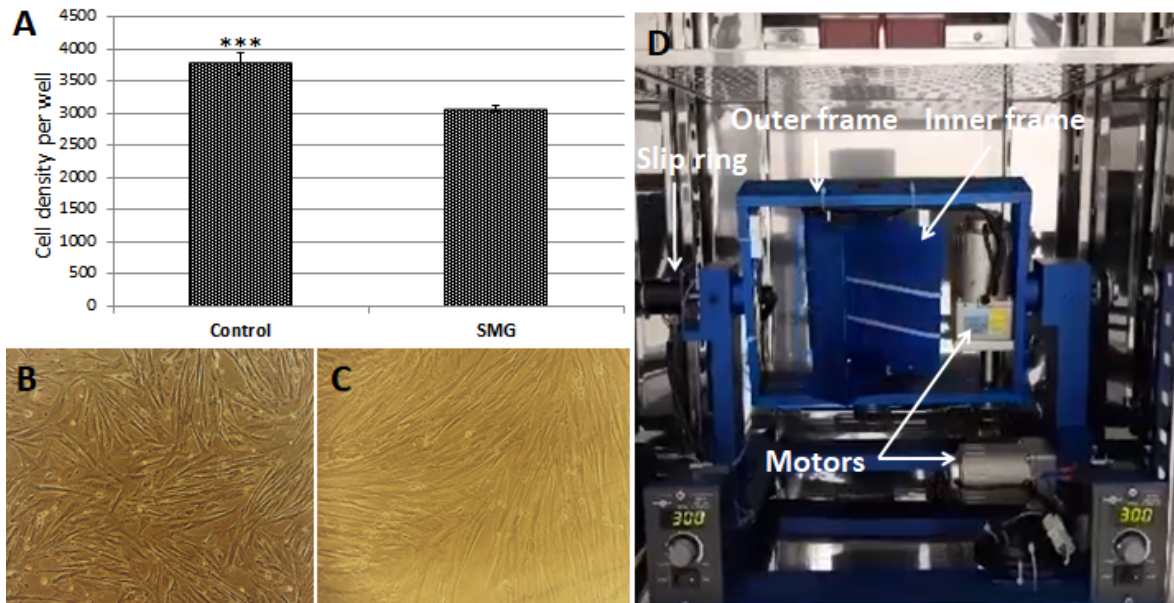


Figure 1. The analysis of WST-1 assay. A. Cell density/well. B,C: MSCs from control group and SMG group. D: 3D clinostat model. ***P < 0.001, Control group vs. SMG group. Magnification: 100x

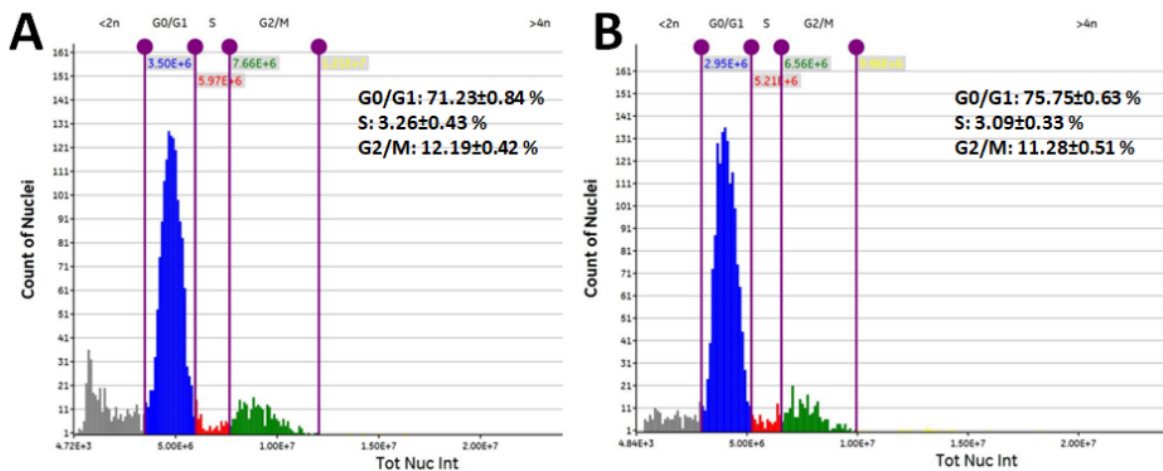


Figure 2. The cycle distribution of MSCs. A: The cycle progress of MSCs from control group, B: The cycle progress of MSCs from SMG group.

The effect of SMG on MSC cycle progression was evaluated by Cell Cycle App of Cytell Fluorescence Microscope. As seen in Figure 2, the MSC ratio of G0/G1 phase from SMG group ($75.75 \pm 0.63\%$) was higher than that of the control group ($71.23 \pm 0.84\%$) ($P < 0.01$). There was no difference in MSC

percentage in S phase between the SMG group ($3.09 \pm 0.33\%$) and control group ($3.26 \pm 0.43\%$). The MSC ratio of G2/M phase from SMG group ($11.28 \pm 0.51\%$) was similar to the control group ($12.19 \pm 0.42\%$). However, the total MSC ratio of SMG group in S phase and G2/M phase was lower than that of the control

group ($P < 0.01$).

The qRT-PCR was applied to estimate the transcript expression of cell cycle-related genes of MSCs. The results from Figure 3 demonstrated that the Cdk2 transcript expression of MSCs from SMG group was lower than that of the control group ($P < 0.001$). The down-regulation of Cdk6 transcript expression was observed in MSCs from SMG group, comparing to the control group ($P < 0.05$). In addition, MSCs of SMG group showed lower Cyclin A transcript expression than the control group.

The analysis of flow cytometry data indicated that there was no difference in viability ratio of MSCs from control group ($93.20 \pm 0.35\%$) and SMG group ($92.85 \pm 1.15\%$) (Figure 4). The total apoptotic ratio (early apoptosis and late apoptosis) of MSCs from SMG group was similar to the control group. The qRT-PCR was also used to estimate the transcript expression of Bcl2 and Bax of MSCs. The results from Figure 5 indicated that no statistically significant difference in Bcl2 and Bax transcript expression of MSCs from control group and SMG group.

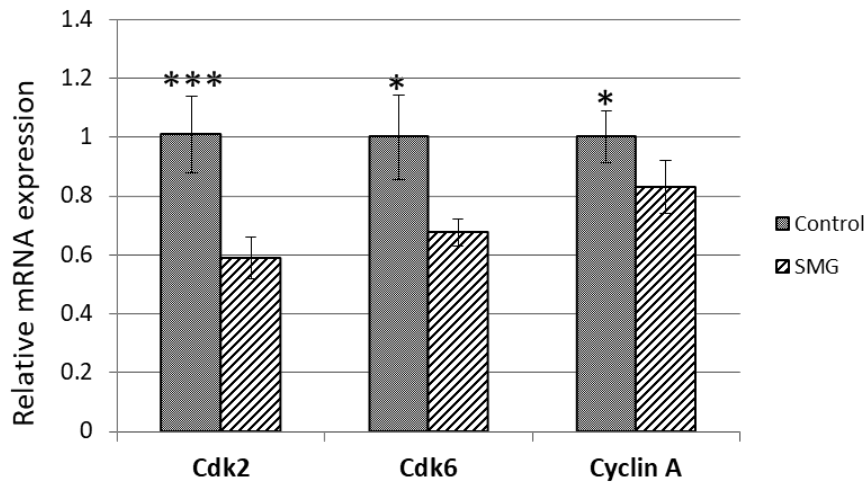


Figure 3. Detection of cell cycle-related genes level by qRT-PCR. *** $P < 0.001$, Control group vs. SMG group, * $P < 0.05$, Control group vs. SMG group.

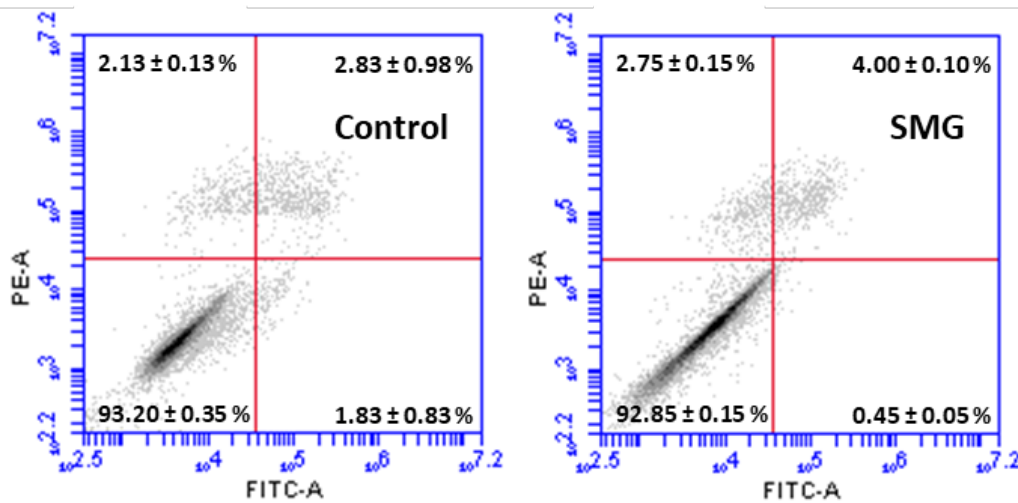


Figure 4. Flow cytometry analysis of apoptosis in MSCs.

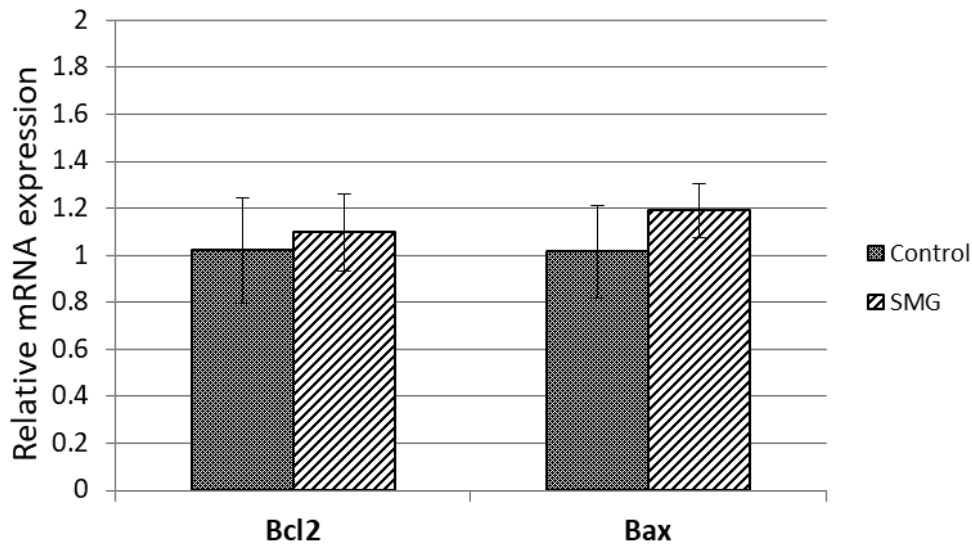


Figure 5. Detection of Bcl2 and Bax transcript expression by qRT-PCR.

The effects of gravitational force have been shown in previous studies, by demonstrated the morphological changes or determination of cell properties (Hoson *et al.*, 1999; Herranz and Medina, 2014; Kim *et al.*, 2017a; Imura *et al.*, 2018). Several cell lines such as adipocytes, monocytes, human osteoblasts, mouse embryonic stem cells, rat MSCs have been used as model for simulated microgravity researches (Yuge *et al.*, 2003; Huang *et al.*, 2009; Kawahara *et al.*, 2009; Meloni *et al.*, 2011). In this study, we found that the density of MSCs in SMG group was lower than the control group after 3 days of culture. This proved that induced SMG conditions induced the decrease the MSC growth. This result consists to study of research of Yan *et al.* (2015). The results of the cell cycle analysis showed that the MSC ratio in G0/G1 phase in SMG group was higher than the control group, which showed that the condition of SMG induced MSCs to enter the arrest phase in the cell cycle. In addition, the percentage of MSCs in S phase and G2/M phase of SMG group was smaller than the control group, this showed that SMG condition induced a reduction in MSC division process.

The cell cycle was controlled by Cyclin dependent kinases (Cdk) in which Cdk2 plays

an essential role in the transition of S phase and distribution of S phase initiation (van den Heuvel, Harlow, 1993; Aleem *et al.*, 2005). In the present study, the Cdk2 transcript expression of MSCs from SMG group was lower than that of the control group, suggesting that the SMG condition could induce the down-regulation Cdk2, leading to the reduces MSC ratio of S phase. On the other hand, Cdk6 is a member of Cyclin dependent kinases which plays a crucial role for G1 phase progression and G1/S transition (Meyerson *et al.*, 1992; Kollmann *et al.*, 2013). In this work, the Cdk6 transcript expression of MSCs from SMG group was lower than control group, revealing that SMG condition could induce the decrease of Cdk6 transcript expression, revealing that MSC ratio was diminished from G1 phase to S phase. The current study also assessed the Cyclin A transcript expression which plays a critical role for S phase (De Boer *et al.*, 2008). Our result indicated that SMG condition induced a down-regulation of Cyclin A transcript expression. These results supported for the increase of G0/G1 phase and reduction of S and G2/M phase in MSCs under SMG condition, this correlated to the decrease of MSC density.

CONCLUSION

SMG condition exhibited no effects on MSC viability. However, SMG-induced MSCs exposed the reduced proliferation by down-regulating the transcript expression of cell cycle-related genes. This resulted in the inducing of MSCs to the arrest phase in the cell cycle progression.

Acknowledgements: *This work was supported by grant VT-CB.15/18-20 of Space Science and Technology Program from Vietnam Academy of Science and Technology.*

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SỰ ỨC CHẾ TĂNG SINH CỦA TẾ BÀO GỐC TRUNG MÔ TRONG ĐIỀU KIỆN VI TRỌNG LỰC MÔ PHÒNG

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TÓM TẮT

Nghiên cứu đánh giá ảnh hưởng của điều kiện vi trọng lực mô phỏng (SMG) lên dòng tế bào gốc trung mô (MSCs). Hệ thống clinostat 3D được sử dụng để tạo ra môi trường vi trọng lực mô phỏng. Kết quả nghiên cứu cho thấy mật độ của các tế bào MSCs ở nhóm đối chứng cao hơn so với nhóm SMG. Phân tích chu kỳ tế bào cũng cho thấy tỉ lệ các tế bào MSCs đi vào pha G0/G1 ở nhóm SMG cao hơn so với nhóm đối chứng, trong khi tỉ lệ MSCs đi vào pha S và pha G2/M ở nhóm SMG lại thấp hơn nhóm đối chứng. Phương pháp Realtime qRT-PCR được dùng để đánh giá biểu hiện phiên mã các gen liên quan đến chu kỳ tế bào bao gồm Cyclin-dependent kinase 2 (Cdk2), Cyclin-dependent kinase 6 (Cdk6) và Cyclin A. Kết quả phân tích Realtime qRT-PCR cho thấy

các gen này đều giảm biểu hiện ở nhóm tế bào cảm ứng vi trọng lực (SMG). Phương pháp flow cytometry được thực hiện để xác định tỉ lệ chết theo chương trình của tế bào MSCs trong hai nhóm thí nghiệm. Kết quả cho thấy không có sự khác biệt mang ý nghĩa thống kê về tỉ lệ tế bào chết theo chương trình giữa nhóm SMG và nhóm đối chứng. Phân tích Realtime qRT-PCR cũng cho thấy giữa hai nhóm thí nghiệm có mức độ biểu hiện tương đương hai gen liên quan đến sự chết theo chương trình là Bcl2 và Bax.

Từ khóa: *chết theo chương trình, chu kỳ tế bào, clinostat 3D, tế bào gốc trung mô, vi trọng lực mô phỏng*