Assessment of cell cycle and viability of magnetic levitation assembled cellular structures

1st Muge Anil-Inevi
Department of Bioengineering
Izmir Institute of Technology
Izmir, Turkey
mugeanil@iyte.edu.tr

2nd Yagmur Ceren Unal
Department of Molecular Biology and Genetics
Izmir Institute of Technology
Izmir, Turkey
yagmurunal@iyte.edu.tr

3rd Sena Yaman
Department of Bioengineering
Izmir Institute of Technology
Izmir, Turkey
senaymn@gmail.com

4th H.Cumhur Tekin
Department of Bioengineering
Izmir Institute of Technology
Izmir, Turkey
cumhurtekin@iyte.edu.tr

5th Gulistan Mese
Department of Molecular Biology and Genetics
Izmir Institute of Technology
Izmir, Turkey
gulistanmese@iyte.edu.tr

6th Engin Ozcivici
Department of Bioengineering
Izmir Institute of Technology
Izmir, Turkey
enginozcivici@iyte.edu.tr

***Abstract*— Label-free magnetic levitation is one of the most recent Earth-based *in vitro t*echniques that simulate the microgravity. This technique offers a great opportunity to biofabricate scaffold-free 3-dimensional (3D) structures and to study the effects of microgravity on these structures. In this study, self-assembled 3D living structures were fabricated in a paramagnetic medium by magnetic levitation technique and effects of the technique on cellular health was assessed. This magnetic force-assisted assembly system applied here offers broad applications in several fields, such as space biotechnology and bottom-up tissue engineering.**

Keywords—biofabrication; magnetic levitation; simulated microgravity; 3D culture.

I. INTRODUCTION

Mechanical forces are one of the important stimuli that affect the cellular fate [1-4]. Specifically, these forces can be important determinants of cell cycle and cell viability [5, 6]. The reduction of mechanical forces acting on cells as in spaceflight causes various impairments such as muscle and bone loss [7-10]. Simulated microgravity platforms have improved our knowledge on *in vitro* biological effects of the microgravity on Earth [11]. Label-free magnetic levitation technique offers an alternative to rotation-based microgravity bioreactors by eliminating the additional mechanical forces caused by rotation on cells [12]. In order to eliminate the high magnetic gradient requirement for levitation of the diamagnetic subjects, systems capable of levitation between two permanent magnets (<1 Tesla) have been described by using a suspending medium with high molar magnetic susceptibility [13, 14]. This system, which was previously applied for density-based separation of cells [15-17], has been recently adopted for the biofabrication of 3D cultures [18-20]. Biofabrication and maintenance of 3D living structures are of great importance in biology research to provide a more physiologically relevant cellular environment [21-23]. Label-free magnetic levitation presents an inexpensive and easy-to-use system that enables formation of scaffold-free 3D cultures and *in-situ* investigation of the biological effects of microgravity environment [24, 25]. Thus, this system can eliminate both the high cost in space-based experimentations [26, 27] and biomaterial-related challenges in scaffold-based approaches [28, 29]. In this study, we applied label-free magnetic levitation technique for biofabrication and maintenance of 3D living structures, and showed suitability of the technique in terms of cellular health.

II. METHODS

A. Magnetic Levitation Device

Magnetic levitation device is composed of two neodymium magnets (grade: N52; l: 50 mm, w: 2 mm, h: 5 mm) with like poles facing each other (distance: 1,5 mm), a glass micro-capillary channel between the magnets, tilted side mirrors to visualize levitated cells in the capillary channel under a microscope (Fig. 1a, b) [24]. The holders to assemble these device components are printed by a 3D printer. Diamagnetic cells suspended in a paramagnetic medium are pushed from high magnetic field region to low magnetic field. In the magnetic field gradient (Fig. 1c), cells are balanced at a position where the sum of magnetic force (F_{mag}, Eq. 1) and gravitational force (F_g, Eq. 2) is zero, and levitated.

978-1-7281-8073-1/20/$31.00 ©2020 IEEE

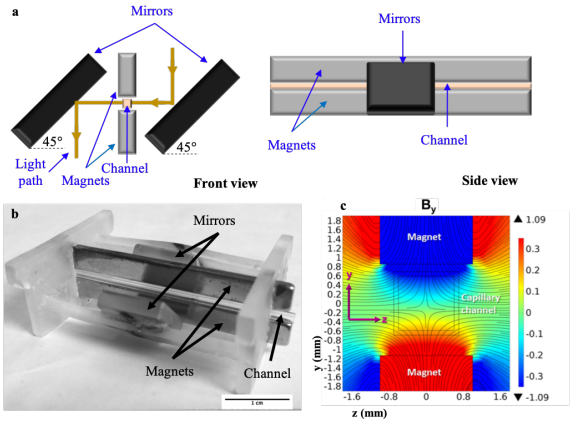

Fig. 1 Magnetic levitation system. (a) Illustration of front and side view of the system. (b) Photograph of the magnetic levitation device (Scale bar, 100 µm). (c) Magnetic induction (y component, B_y) between the neodymium magnets via Finite Element Methodology. Streamlines: total magnetic induction (B_y+B_z) [24].

$$\overrightarrow{F_{mag}} = \frac{V.(x_{cell}-x_{medium})}{\mu_0}(\vec{B}.\nabla)\vec{B} \quad (1)$$

$$\overrightarrow{F_g} = V\Delta\rho g \quad (2)$$

Here, V: volume of cell, x_{cell}-x_{medium}: magnetic susceptibility difference between cells and the surrounding medium, μ_o: magnetic permeability of free space (1.2566×10^{-6} kg·m·A^{-2}·s^{-2}), B: magnetic flux density, $\Delta\rho$: density difference between cell and the surrounding medium and g: vector of gravity.

B. Cell Culture

D1 ORL UVA (bone marrow stem cell line) cells were cultured in DMEM (Gibco), supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin, and grown in a humidified 37 °C incubator with 5% CO_2. The growth medium was changed every 2-3 days and the cells were passaged when they reached 80-90% confluence (every four to six days.).

C. Cell Viability Assay

Cells were seeded at a concentration of 10^4 cells/well in a 96-well plate. After 48 h, cells were exposed to five different Gd-based paramagnetic solutions; gadobutrol (Gd-BT-DO3A, Gadavist®, Bayer), gadopentetate dimeglumine (Gd-DTPA, Magnevist®, Bayer), gadodiamide (Gd-DTPA-BMA; OmniscanTM, GE Healthcare), gadoterate meglumine (Gd-DOTA; Dotarem®, Guerbet) and gadobenate dimeglumine (Gd-BOPTA; Multihance®, Bracco). Gd-based solutions were added to the culture medium at increasing concentrations (0, 10, 25, 50, 100 and 200 mM). Cell viability was measured at 24th h of culture with thiazolyl blue tetrazolium bromide (MTT) assay. MTT reagent (0.5 mg/ml) was added to the samples and the cells were incubated at 37 °C for 3 h in the dark. The media was removed, formazan crystals were dissolved in 100 µl DMSO. Optical density measurements were performed at 570 nm with a reference wavelength of 690 nm (Thermo Scientific Multiskan Go).

D. Live/Dead Assay

For assessment of cell viability, D1 ORL UVA cells were cultured in 2D culture and 3D magnetic levitation cultures (100 mM Gd^{3+}, 5000 cells/capillary channel) for 3 days. Cell viability were assessed by live/dead assay (calcein-AM/propidium iodide, Sigma Aldrich) and visualized under the fluorescence microscope (Olympus IX-83).

E. Biofabrication and Culture of 3D Living Structures under Microgravity

D1 ORL UVA cells were harvested by trypsinization and suspended in paramagnetic medium (100 mM Gd^{3+}) at a concentration of 10^6 cells/mL (50,000 cells per capillary) and levitated in the magnetic levitation device. In 2D culture group, cells were inoculated in a medium containing 100 mM Gd^{3+} in 48-well cell culture dishes at a concentration of $2{,}5x10^5$ cells/well. All cells were cultured for 2 or 24 h at 37 °C and 5% CO_2 in a humidified atmosphere and observed under the inverted microscope (Olympus IX-83).

F. Flow Cytometry

In the levitation culture groups, five levitation culture samples were pooled and thus the number of cells in one sample was increased in sufficient number for cell cycle analysis. To determine the cell cycle stages, cells of all experimental groups were stained with Propidium iodide (PI) and measured by flow cytometry. Cells were harvested from magnetic levitation culture and 2D culture. After centrifugation, cell pellets were dissolved in 1ml cold PBS and 4 ml cold 100% EtOH and stored at -20ºC. For analysis, the cells were centrifuged and dissolved in 2µL RNase A (200 µg/mL) and 200µL 0.1% Triton X-100 PBS, and incubated at 37ºC for 30 minutes. After the incubation, 20µl PI (1mg / ml) was added and kept in the dark for 15 minutes and then measurements were performed with BD software in BD FACS Canto flow cytometry.

III. Results and Discussion

In order to quantify the effect of several Gd^{3+} chelates in terms of chemical content and structure on cell viability, D1 ORL UVA cells were cultured with different paramagnetic medium at increasing concentrations (0, 10, 25, 50, 100 and 200 mM) in 2D and cell viability were measured by MTT assay (Fig. 2a). At the end of 24 h-culture, it was determined that only Gd-BT-DO3A agent did not reduce the cell viability statistically significantly ($p > 0.05$) at any concentration. In our previous study, long-term levitation culture of cells was tested at all concentrations of this contrast agent, and it was shown that 100 mM concentration of the agent was sufficient to maintain 3D structures in a levitated position during the culture [24]. The cells were then cultured for 3 days in a paramagnetic medium obtained with 100 mM Gd-BT-DO3A both in 2D culture and in 3D levitation culture to observe the viability of the cells (Fig. 2b). It was observed that almost all of the cells were alive in both cultures. These results suggest that ligand structure is strongly associated with the release of free Gd^{3+} and thus toxic effect of

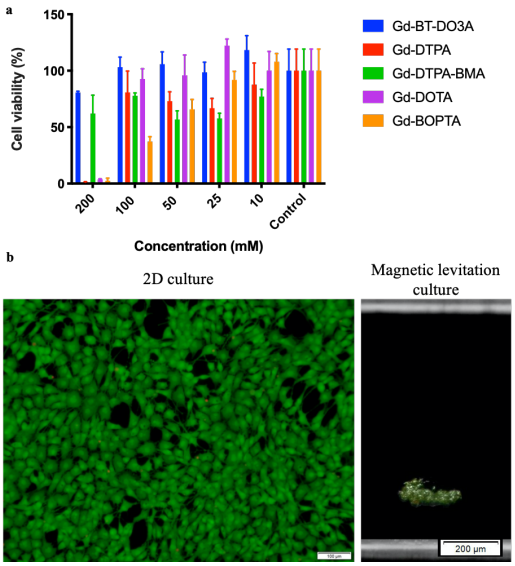

Fig. 2 Viability of D1 ORL UVA cells in paramagnetic medium. (a) Cell viability after 24 h-exposure to five different contrast agents at increasing concentrations (0, 10, 25, 50, 100 and 200 mM). Cell viability was measured with MTT assay. (b) Live-dead analysis of cells cultured with 100 mM Gd-containing medium in 2D culture or magnetic levitation culture for 3 days. Scale bar: 200 µm.

agent, and Gd-BT-DO3A required to levitate cells are suitable for long term cell culture.

D1 ORL UVA cells were cultured in both 2D and 3D magnetic levitation culture for 2 and 24 h (Fig. 3) and cell cycle ratios in the population was analyzed to test the effects of magnetic levitation culture on cell cycle stages (Fig. 4). No statistically significant difference was shown between the rates of cells in the G1, G2 and S phases observed in cells cultured with magnetic levitation for 2 or 24 h and that of 0 h control

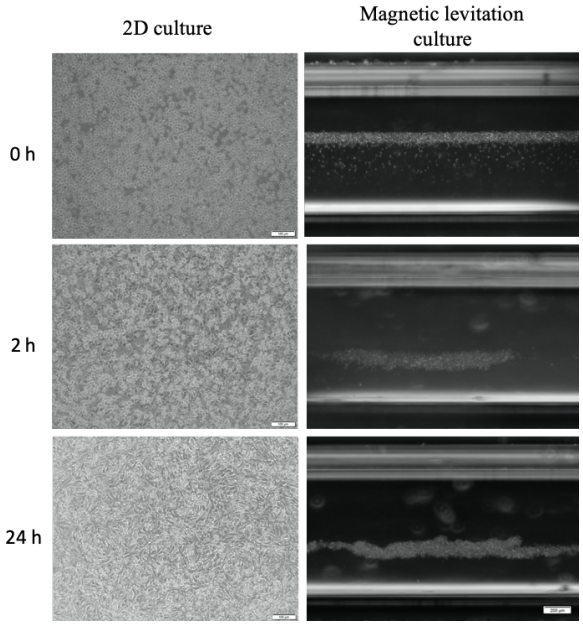

Fig. 3 Micrographs of D1 ORL UVA cells cultured with Gd-BT-DO3A (100 mM Gd^{+3}) in 2D and 3D magnetic levitation for 0, 2 and 24 h. Scale bar: 100 µm for 2D culture, 200 µm for magnetic levitation culture.

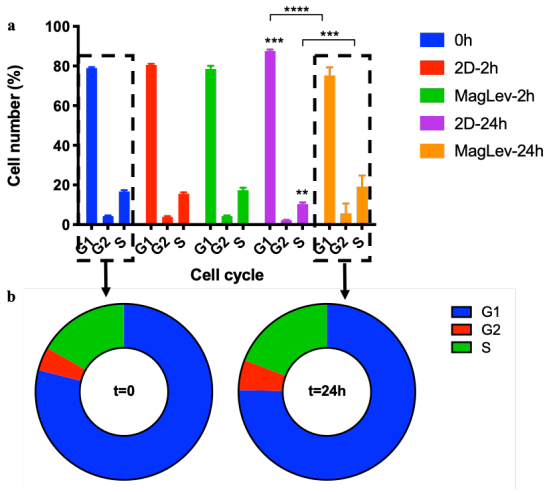

Fig. 4 Cell cycle distribution in D1 ORL UVA cells with MagLev culture. (a) Cell cycle distribution in the population (%) at 0, 2 and 24 hours of culture, suspended with medium containing 100 mM Gd^{+3} and cultured in 2D culture or magnetic levitation device (50,000 cells/capillary). It is presented as the mean with deviation (± SD). The data were analyzed using two-way ANOVA and Dunnett's multiple comparisons test (for comparison with 0 h control) and Tukey's multiple comparisons test (for comparison with 2D-MagLev control). Statistical significance was defined as $P < 0.05$. (b) Cell-cycle structure of 24-h MagLev culture group (right) and 0-h control group (left).

group. In addition, as a result of 24-h culture, it was observed that cells cultured in magnetic levitation had less cell ratio in G1 stage and more cells in S stage compared to 2D culture. That cells cultured in magnetic levitation do not cause a decrease in the S phase of the cell cycle might be interpreted as chemical and mechanical effects of magnetic levitation do not reduce the number of actively cycling cells. We have also successfully applied this biofabrication and culture system suitable for cell health to produce 3D cocultures with different patterns [24, 25].

IV. Conclusions

Mechanical stimuli exert a remarkable impact on cell biology. Label-free magnetic levitation technology provides a system that is cost-effective and allows real-time imaging to simulate absence of gravitational forces on Earth. This technique further allows biofabrication and culture of scaffold-free living 3D structures. Non-cytotoxic nature of the method allows it to be applied in numerous fields several fields, such as bottom-up tissue engineering and basic and preclinical research.

V. Acknowledgment

Financial support by The Scientific and Technological Research Council of Turkey (215S862) is gratefully acknowledged.

References

[1] D. Li, J. Zhou, F. Chowdhury, J. Cheng, N. Wang, and F. Wang, "Role of mechanical factors in fate decisions of stem cells," *Regenerative medicine,* vol. 6, no. 2, pp. 229-240, 2011.

[2] E. Ozcivici *et al.*, "Mechanical signals as anabolic agents in bone," *Nature Reviews Rheumatology,* vol. 6, no. 1, p. 50, 2010.

[3] E. Özçivici, "Effects of spaceflight on cells of bone marrow origin," *Turkish Journal of Hematology,* vol. 30, no. 1, p. 1, 2013.

[4] O. Baskan, O. Karadas, G. Mese, and E. Ozcivici, "Applicability of Low-Intensity Vibrations as a Regulatory Factor on Stem and Progenitor Cell Populations," *Current Stem Cell Research & Therapy,* 2019.

[5] M. Olcum and E. Ozcivici, "Daily application of low magnitude mechanical stimulus inhibits the growth of MDA-MB-231 breast cancer cells in vitro," *Cancer cell international,* vol. 14, no. 1, p. 102, 2014.

[6] O. Baskan, G. Mese, and E. Ozcivici, "Low-intensity vibrations normalize adipogenesis-induced morphological and molecular changes of adult mesenchymal stem cells," *Proceedings of the Institution of Mechanical Engineers, Part H: Journal of Engineering in Medicine,* vol. 231, no. 2, pp. 160-168, 2017.

[7] M. P. Nagaraja and D. Risin, "The current state of bone loss research: data from spaceflight and microgravity simulators," *Journal of cellular biochemistry,* vol. 114, no. 5, pp. 1001-1008, 2013.

[8] T. Ohira, F. Kawano, T. Ohira, K. Goto, and Y. Ohira, "Responses of skeletal muscles to gravitational unloading and/or reloading," *The Journal of Physiological Sciences,* vol. 65, no. 4, pp. 293-310, 2015.

[9] E. Ozcivici and S. Judex, "Trabecular bone recovers from mechanical unloading primarily by restoring its mechanical function rather than its morphology," *Bone,* vol. 67, pp. 122-129, 2014.

[10] S. Judex, W. Zhang, L. R. Donahue, and E. Ozcivici, "Genetic and tissue level muscle-bone interactions during unloading and reambulation," *Journal of musculoskeletal & neuronal interactions,* vol. 16, no. 3, p. 174, 2016.

[11] M. Anil-Inevi, O. Sarigil, M. Kizilkaya, G. Mese, H. C. Tekin, and E. Ozcivici, "Stem Cell Culture Under Simulated Microgravity," 2020.

[12] B. E. Hammer, L. S. Kidder, P. C. Williams, and W. W. Xu, "Magnetic levitation of MC3T3 osteoblast cells as a ground-based simulation of microgravity," *Microgravity science and technology,* vol. 21, no. 4, p. 311, 2009.

[13] K. A. Mirica, S. S. Shevkoplyas, S. T. Phillips, M. Gupta, and G. M. Whitesides, "Measuring densities of solids and liquids using magnetic levitation: fundamentals," *Journal of the American Chemical Society,* vol. 131, no. 29, pp. 10049-10058, 2009.

[14] N. G. Durmus *et al.*, "Magnetic levitation of single cells," *Proceedings of the National Academy of Sciences,* vol. 112, no. 28, pp. E3661-E3668, 2015.

[15] O. Sarigil, M. Anil-Inevi, E. Yilmaz, G. Mese, H. C. Tekin, and E. Ozcivici, "Label-free density-based detection of adipocytes of bone marrow origin using magnetic levitation," *Analyst,* vol. 144, no. 9, pp. 2942-2953, 2019.

[16] O. Sarigil *et al.*, "Application of magnetic levitation induced weightlessness to detect cell lineage," in *2019 9th International Conference on Recent Advances in Space Technologies (RAST)*, 2019: IEEE, pp. 933-935.

[17] K. Delikoyun, S. Yaman, M. Anil-Inevi, E. Ozcivici, and H. C. Tekin, "Cell Separation with Hybrid Magnetic Levitation-Based Lensless Holographic Microscopy Platform," in *2019 Medical Technologies Congress (TIPTEKNO)*, 2019: IEEE, pp. 1-4.

[18] A. Tocchio *et al.*, "Magnetically guided self-assembly and coding of 3D living architectures," *Advanced Materials,* vol. 30, no. 4, p. 1705034, 2018.

[19] M. Anil-Inevi, E. Yilmaz, O. Sarigil, H. C. Tekin, and E. Ozcivici, "Single cell densitometry and weightlessness culture of mesenchymal stem cells using magnetic levitation," in *Stem Cell Nanotechnology*: Springer, 2019, pp. 15-25.

[20] S. Yaman, M. Anil-Inevi, E. Ozcivici, and H. C. Tekin, "Magnetic force-based microfluidic techniques for cellular and tissue bioengineering," *Frontiers in bioengineering and biotechnology,* vol. 6, p. 192, 2018.

[21] A. Atala, "Engineering tissues, organs and cells," *Journal of tissue engineering and regenerative medicine,* vol. 1, no. 2, pp. 83-96, 2007.

[22] E. Yildiz-Ozturk, S. Gulce-Iz, M. Anil, and O. Yesil-Celiktas, "Cytotoxic responses of carnosic acid and doxorubicin on breast cancer cells in butterfly-shaped microchips in comparison to 2D and 3D culture," *Cytotechnology,* vol. 69, no. 2, pp. 337-347, 2017.

[23] M. Anil-Inevi, P. Sağlam-Metiner, E. C. Kabak, and S. Gulce-Iz, "Development and verification of a three-dimensional (3D) breast cancer tumor model composed of circulating tumor cell (CTC) subsets," *Molecular Biology Reports,* vol. 47, no. 1, pp. 97-109, 2020.

[24] M. Anil-Inevi *et al.*, "Biofabrication of in situ self assembled 3D cell cultures in a weightlessness environment generated using magnetic levitation," *Scientific reports,* vol. 8, no. 1, pp. 1-10, 2018.

[25] M. Anil-Inevi *et al.*, "Biofabrication of cellular structures using weightlessness as a biotechnological tool," in *2019 9th International Conference on Recent Advances in Space Technologies (RAST)*, 2019: IEEE, pp. 929-931.

[26] M. Böhmer and E. Schleiff, "Microgravity research in plants: A range of platforms and options allow research on plants in zero or low gravity that can yield important insights into plant physiology," *EMBO reports,* vol. 20, no. 7, p. e48541, 2019.

[27] K. Delikoyun, E. Cine, M. Anil-Inevi, M. Ozuysal, E. Ozcivici, and H. C. Tekin, "Lensless digital in-line holographic microscopy for space biotechnology applications," in *2019 9th International Conference on Recent Advances in Space Technologies (RAST)*, 2019: IEEE, pp. 937-940.

[28] D. F. Williams, "Challenges with the development of biomaterials for sustainable tissue engineering," *Frontiers in bioengineering and biotechnology,* vol. 7, p. 127, 2019.

[29] M. Anil, D. Ayyildiz-Tamis, S. Tasdemir, A. Sendemir-Urkmez, and S. Gulce-Iz, "Bioinspired materials and biocompatibility," in *Emerging Research on Bioinspired Materials Engineering*: IGI Global, 2016, pp. 294-322.