Journal of Sciences, Islamic Republic of Iran 32(2): 105 - 114 (2021) http://jsciences.ut.ac.ir
University of Tehran, ISSN 1016-1104

The Impacts of Simulated Microgravity on The Cell Viability and Claudin-1 and Claudin-3 Expression of MCF-7 Breast Cancer Cells

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Received: 24 October 2020 / Revised: 22 January 2021 / Accepted: 3 February 2021

Abstract

It has been believed that microgravity directly can alter the structure, morphology, and function of biosystems and numerous research have been performed to recognize these alterations. Claudin proteins are the tight junctions' main components. Additionally, they are crucial for the protection of the differentiated state of epithelial cells as well as for cell-cell interaction. This study aimed to explore the probable correlation between the claudin-1 and claudin-3 expression and microgravity condition. Additionally, examined the impacts of microgravity condition on cell morphology and viability. The gene expression in MCF-7 cells were assessed by real-time quantitative RT PCR. Afterward, the morphology and cellular viability of the cells were evaluated by an inverted microscope, MTT assay, and flow cytometry analysis. After 72 h of simulated microgravity, the claudin-1 and claudin-3 expression increased significantly (P<0.05). Also, MCF-7 cells after 72 h exposure to microgravity simulation comprised rounded cells, which were grouped and linked to each other making multicellular spheroids. However, microgravity simulation after 24 or 72 h did not have a remarkable effect on the viability of cells. The consequence of this research lied in the fact that simulated microgravity could not be a direct cure for breast cancer treatment. However, microgravity research can offer a unique *in vitro* tool to explore biomechanical effects in the biology of cancer. The findings obtained from this investigation can open fascinating research lines in astrobiology, biophysics, and cancer biology and can be utilized to improve survivability and life quality for malignancy patients.

Keywords: Claudin-1 Gene; Claudin-3 Gene; Simulated Microgravity; MTT assay; Flow cytometry analysis.

Introduction

Advances in technology have offered amazing opportunities for the human to travel more rapidly on or near the Earth surface. The primary goals of space travel are the search for life, planetary exploration, and more crucially safe return to Earth. Humans on Earth are adapted to the constant gravitational force (9.8 m/s^2) [1].

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Nevertheless, in space, gravity is much weaker than on Earth which is known as microgravity. Presently, studies on the growth and development of cells exposed to microgravity, as biophysical force, is a hot topic in cell biology and astronauts' health [2, 3]. It has been known that gravity affects lots of physical issues, for instance, viscosity, diffusion process, and shear forces, therefore, it could affect some general features of the biosystems undeniably [2, 3]. The experiences of cosmonauts and astronauts over the past four decades have proven that microgravity can induce countless physiological disruptions such as vision loss, sarcopenia (a loss of muscle mass), osteopenia (low bone density), diminished neurological responses, decreased renal function, and a weakened or compromised immune system [4].

According to the literature, microgravity can induce variations in the function, morphology, and growth of the cells, directly [4, 5]. The relationship between tumorigenesis and microgravity condition has attracted special interest for scientifics. It has been shown that microgravity can induce gene expression alterations, proliferation, signal transduction as well as morphology in several malignant cells by affecting the mechanical tumor micro-environment [6, 7]. Breast cancer, as one of the most frequent types of female cancer, could be categorized into five subtypes, involving HER2 (human epidermal growth factor receptor 2), luminal A, basal, claudin-low (CL), and luminal B [6, 7]. The MCF-7 cells belong to the Luminal A subtype, which is identified through the lack of HER2 expression. Until now, there have been various studies investigating the effects of microgravity on breast cancer cell lines. Strube *et. al.* exposed that simulated microgravity could increase BRCA1 (breast cancer antigen 1) and VCAM1 (vascular cell adhesion molecule 1) expression and decrease KRAS (Kirsten ras oncogene) and VIM (vimentin) expression significantly. They also proved that simulated microgravity could not alter the expression of MMP13 (matrix metalloproteinase 13), TP53 (tumor protein 53), MAPK1 (mitogen-activated protein kinase 1), and PTEN (phosphatase and tensin homolog) [8]. Chen *et al*. indicated that simulated microgravity did not disturb the overall growth rate of MDA-MB-231 cells and MCF-7 cells. However, the authors described a noticeable adherent cells accumulation in the synthesis phase of the cell cycle [9]. Masiello *et. al.* highlighted that simulated microgravity condition causes diverse morphological changes in MDA-MB-231 cells [10]. Kopp *et. al.* suggested that once MCF-7 cells transit to a 3D growth on the random positioning machine (RPM), HMOX-1 (heme oxygenase (decycling) 1) and NFκB family members interact on a gene level [11]. CDH1 (cadherin 1) and E-cadherin protein downregulation in MCF-7 cells were also detected under RPM-exposure [12].

Claudin proteins are the tight junctions' main components. Additionally, they are crucial for the protection of the differentiated state of epithelial cells as well as for cell-cell interaction. It has been shown that the expression status of claudin family members varies in various cancer types [13]. In breast carcinoma, the expression of claudin-7 and claudin-1 are downregulated, and the expression of claudin-3 and claudin-4 are upregulated [13]. Akasaka *et. al.* revealed that claudin-1 has anti-apoptotic impacts, and also is involved in the expression regulation and of E-cadherin and β-catenin subcellular localization in MCF-7 cells [14].

Currently, owing to progress in space technology, the chances for humans subjected to microgravity have increased undeniably. Until now, there has been no research about the microgravity impacts on claudin-1 and claudin-3 expression. Hence, this study aimed to explore the probable correlation between the claudin-3 and claudin-1 expression and microgravity condition. Also, investigated the impacts of simulated microgravity condition on cell morphology and cell viability of MCF-7 cells. The findings obtained from this investigation can help to determine whether biological processes such as gene expression and cell morphology are the same in space as they are on earth. Also, this study can help to verify whether microgravity could be an option for tumor treatment or not.

Materials and Methods

Materials

Dimethyl sulfoxide (DMSO) and 3- [4, 5-Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich Chemical Co. (Missouri, United States of America). The MCF-7 cell line (ATCC® HTB-22™) was bought from the National Cell Bank of Pasteur Institute (Tehran, Iran). Fetal bovine serum (FBS), streptomycin, RPMI 1640 medium, penicillin, and Trypsin-EDTA were bought From Gibco (New York, USA). From SinaClon Co. (Tehran, Iran) RNX-Plus Solution for total RNA isolation was acquired. DNaseI was purchase from Fermentas, (Vilnius, Lithuania). Phosphate-buffered saline (PBS) solution (1X) was obtained from Bio-Idea Co. (Tehran, Iran). Annexin FITC kit was bought from IQ Products (Groningen, Netherlands). The Primescript™ RT reagent Kit, the SYBR Green real-time Master Mix kit, and the Cell Amp™ Direct RNA Prep Kit were obtained from Takara Bio Inc. (Shiga,

Japan). Deionized double-distilled water (Barnstead™ Nanopure infinity water purification system (Lake Balboa, California, USA)) was used through all measurements.

Cells and Cell Culture

In RPMI 1640 medium (containing streptomycin and penicillin (5 µg.mL^{-1}), heat-inactivated FBS (10%), and L-glutamine (2 mM)) the MCF-7 cells were cultured. The cancer cells were preserved in an incubator with a humidified atmosphere of 5% CO_2 and 37 °C temperature. This cell line was grown routinely as a monolayer culture.

Microgravity Simulation

Ground-based simulators are utilized to generate altered-gravity conditions and offer cost-effective platforms for gravitational investigation. In this study, the two-dimensional clinostat (2D clinostat; a donation from the United Nations Office at Vienna; the office for outer space affairs (UNOOSA)) was applied for simulating microgravity (Fig. 1). During rotation, this simulator prevents gravity from influencing MCF-7 cells. The major body size of the clinostat was 25 cm×25 cm×25 cm and the control box size of the device was 23 cm×20 cm×11 cm. The clinostat was sterilized through 70% ethanol and UV radiation and placed in a 37 °C incubator.

Preparation of Samples

Various physical factors can disturb the performance of the two-dimensional clinostat: the sample diameter, time sensitivity, the rotation speed, and the clinostat horizontal placement. It has been reported that the centrifugal force amount utilized on the samples was in the order of 10^{-3} g, if the rotation speed was fixed at 10 rpm [15]. Thus, at first, the MCF-7 cells were divided into two groups, comprising one control group (the cancer cells subjected to normal gravity) as well as the experimental group (the MCF-7 cells subjected to simulated microgravity). Consequently, the experimental group (in a 3 mL small shielded tube) was fixed at a 3 cm distance from the center of the clinostat, and the control samples were kept next to the clinostat in the incubator during the time of rotation. The clinostat rotation times were 24 h and 72 h and the speed of rotation was 20 rpm.

cDNA Synthesis and Quantitative real-time RT PCR

The claudin-3 and claudin-1 expression was investigated in all samples (the cells subjected to normal gravity and simulated microgravity) according to the previous method [16]. At first, total RNA was extracted consuming the RNA isolation kit (RNX-Plus) consistent with the manufacturer's recommendations. To wipe out any DNA impurity, total RNAs were treated with RNase-free DNaseI. Purified RNA absorbances at a wavelength of 260 nm (A260) and a wavelength of 280 nm (A280) were ascertained by the UV spectrophotometer, Agilent Technologies (California, USA). Then, the ratio of A260/280 was determined, and its value was 1.9. Agarose gel electrophoresis (1.2%

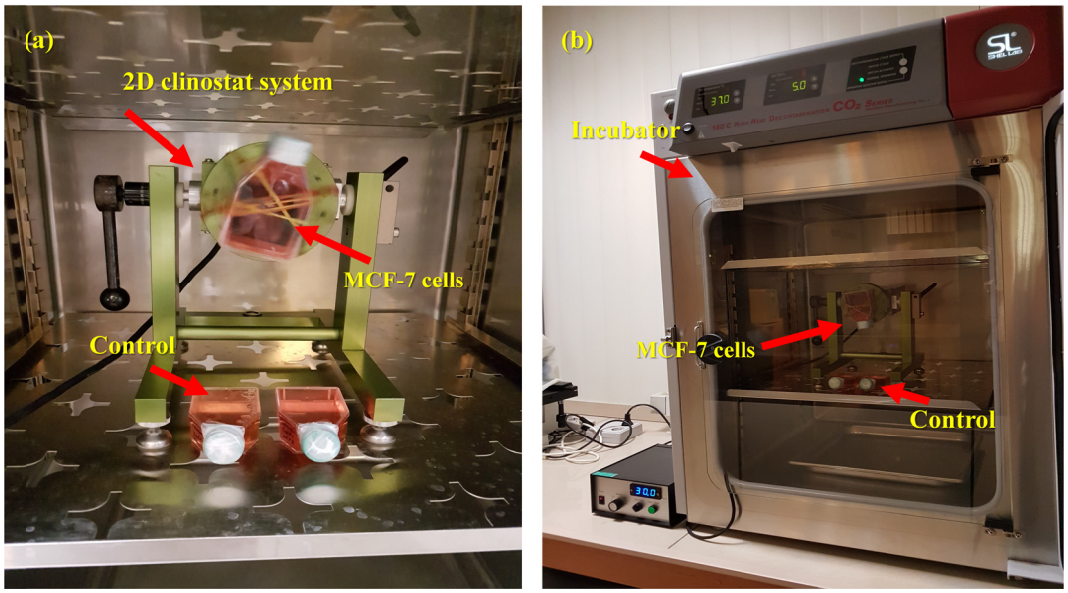

Figure 1. (a, b) The 2D clinostat system and experimental set-up.

(w/v)) of the extracted RNA was analyzed in 1X TBE buffer for 30 min at 80 V, stained with an ethidium bromide (EtBr) solution, and then observed under UV light. It is crucial to mention that although EtBr is a valuable tool, its dangerous properties require disposal procedures as well as special safe handling. Therefore, in this study safety precautions were taken when using ethidium bromide. As displayed in Figure 2a, total RNA

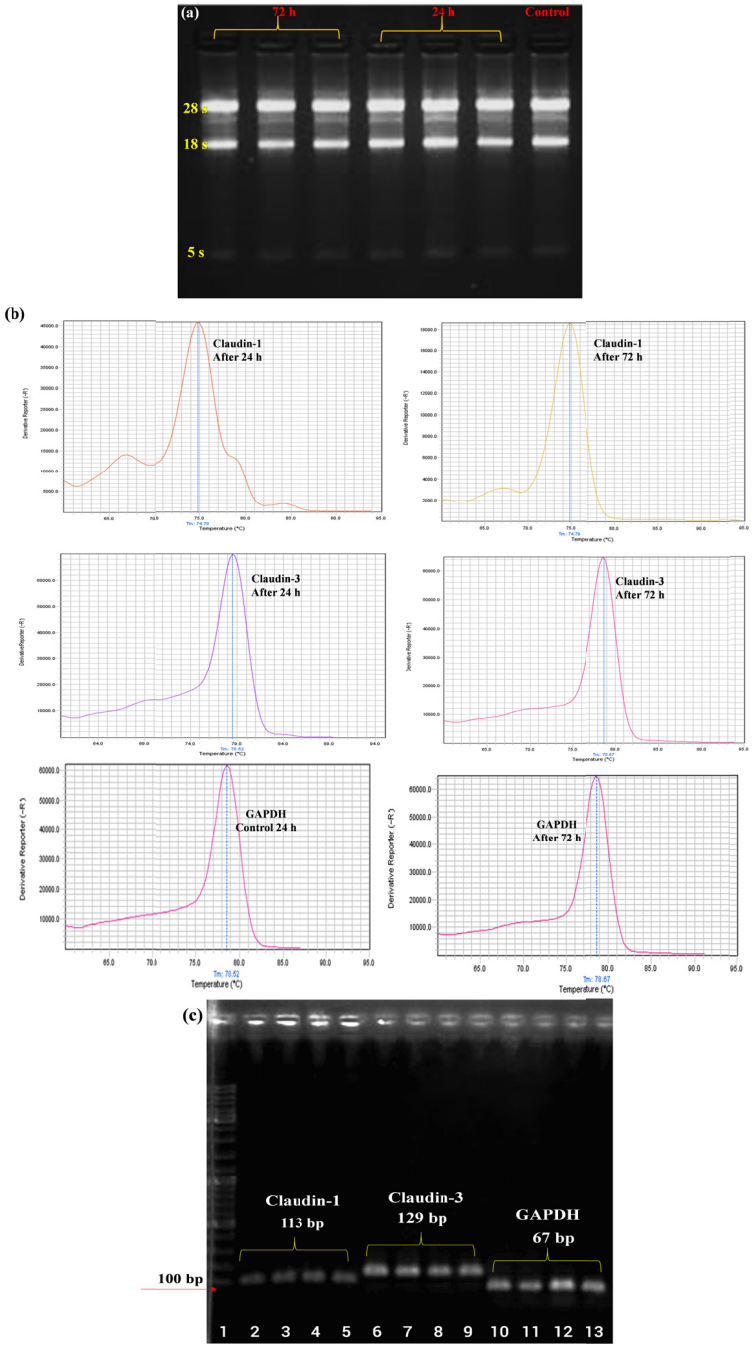

Figure 2. (a) Agarose gel electrophoresis of total RNA extracted from samples. 18S rRNA and 28S rRNA electrophoresis bands for RNA samples extracted from MCF-7 cells. **(b)** Melting temperature (T_m) peak curves of real-time RT-PCR product for GAPDH, claudin-1, and claudin-3. **(c)** Agarose gel electrophoresis of GAPDH, claudin-1, and claudin-3 PCR of samples of synthesized cDNA.

formed sharp and clear 28S and 18S ribosomal bands which was a good indicator that the RNA was not degraded. Afterward, total RNA was utilized for cDNA synthesis employing the Primescript™ RT reagent Kit. Quantitative real-time RT PCR was performed utilizing StepOnePlus Real-Time PCR, Applied Biosystems (California, United States of America) utilizing SYBR Green Master mix (Ex Taq II) with cDNA template (1 µL), forward primer (0.2 µM), reverse primer (0.2 µM), master mix (5 µL), dH_2O, and ROX reference dye II (0.04 µL) to a 10 µL final volume. PCR conditions were: initial denaturation at 95 ºC (2 min); subsequently, 40 cycles of denaturation in 5 sec at 95 ºC, and annealing in 30 sec at 60 ºC. To verify the PCR reaction for apparent of any primer-dimer and validate the specificity of the reaction, PCR melt curves were analyzed. As shown in Fig. 2b, no apparent dimers or non-specific bands were detected in the melting curve analysis, indicating that the designed primers had a high quality and specificity. Also, agarose gel electrophoresis (1.0% (w/v)) of the PCR products was analyzed. As demonstrated in Fig. 2c, claudin-1, claudin-3, and GADPH amplified a single PCR product of the desired size from cDNA pools. Finally, alterations in the fold number were analyzed through the $2^{-\Delta\Delta Ct}$ method, which was normalized via GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene as the housekeeping gene. Gene-specific primers were designed by GenBank (https://www.ncbi.nlm.nih.gov/) and oligo 7 primer analysis software. Then, the designed primers were submitted to the BLAST search versus human genome to verify that the sequences were specific only for the genes of interest. Primers were synthesized via Macrogen Incorporated (Seoul, South Korea) as depicted in Table 1. Two negative controls were included in each PCR reaction, one with no RT treatment (minus-RT) and one with no cDNA template.

Morphology Alterations Study using Phase Contrast Microscope

Observation of morphological variations of cells was performed according to the previous method [17]. The cells were subjected to simulated microgravity and normal gravity. Then, the medium of each cultured line was removed and washed once with 2 ml of cold PBS buffer (pH 7.4, 0.01 M). Subsequently, the MCF-7 cells were photographed utilizing a phase-contrast microscope, Olympus CK40-32PH (Tokyo, Japan).

MTT Cytotoxicity Assay

The MCF-7 cells were subjected to simulated microgravity (for 24 h and 72 h) and then were trypsinized with Trypsin-EDTA and seeded in 96-well plates. Later, MTT (20 µL from 5 $mg.mL^{-1}$ in PBS buffer) was added into each well then incubated at 37 °C for 180 min. Next, the insoluble formazan formed was dissolved in DMSO (100 µl). The OD (optical density) of each well, was determined against reagent blank with Model Expert 96 ELISA reader, Asys Hitech (Salzburg, Austria) at 570 nm. The experiment consists of 4 repeated trials.

Flow cytometry Analyses

The MCF-7 cells were subjected to simulated microgravity for 24 h and 72 h and then cells were gathered by centrifugation at 1000 g for 5 min then washed with PBS twice. Afterward, suspended in Annexin V binding buffer (100 µL). Therefore, MCF-7 cells were double-stained with PI (10 µL) and Annexin V (10 µL) solution (50 $\mu g.mL^{-1}$ in PBS). The MCF-7 cells were maintained in darkness at room temperature (20 min) later examined through Partec PAS III flow cytometry cytometer (Muenster, Germany).

Statistical analysis

Relative expression was evaluated using REST 2009 software (Version 2.0.13). Significant differences were analyzed by t-test of GraphPad Prism Software (Version 8.4.3, San Diego, United States of America). GraphPad Prism also was used for plotting graphs. All of the data were expressed as the mean ± the standard deviation (SD). * exposes a considerable difference. * reveals $P<0.05$ and ** reveals $P<0.01$.

Table 1. Features of designed primers

Genes	Primer sequence (5' to 3')	Amplicon size (bp)
GAPDH		
Forward primer	ACGACCACTTTGTCAAGCTCAT	67
Reverse primer	TCCACCACCCTGTTGCTGTA	
Claudin-1		113
Forward primer	GTGCGATATTTCTTCTTGCAGG	
Reverse primer	TTCGTACCTGGCATTGACTGG	
Claudin-3		129
Forward primer	CTGCTCTGCTGCTCGTGTCC	
Reverse primer	TTAGACGTAGTCCTTGCGGTCGTAG	

Results

Claudin-1 and Claudin-3 Expression Analysis

The real-time RT PCR method was operated to measure the effect of simulated microgravity on the expression of the claudin-3 and claudin-1 gens in MCF-7 cells. As displayed in Figure 3a,b compared to the control group, after 24 h of simulated microgravity the claudin-1 expression did not change, and the claudin-3 expression diminished very slightly (P<0.05). After 72 h of simulated microgravity, the claudin-3 and claudin-1 expression increased significantly (P<0.01). In another word, the claudin-1 and claudin-3 expression didn't come back to the control level after 72 h exposure to simulated microgravity. Taken together, the statistical analysis revealed that there is a significant and clear correlation between claudin-1 and claudin-3 expression and microgravity condition, especially after 72 h exposure.

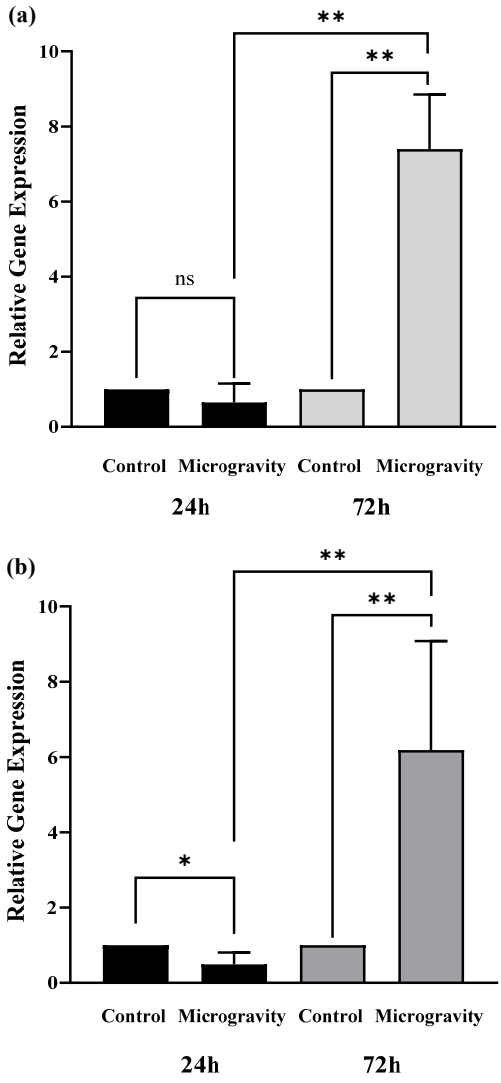

Figure 3. Relative gene expression of **(a)** claudin-1 and **(b)** claudin-3 under simulated microgravity condition after 24 h and 72 h in comparison to the control groups. Values are mean ± standard deviation; ns = non-significant, *indicates P<0.05 and ** indicates P<0.01.

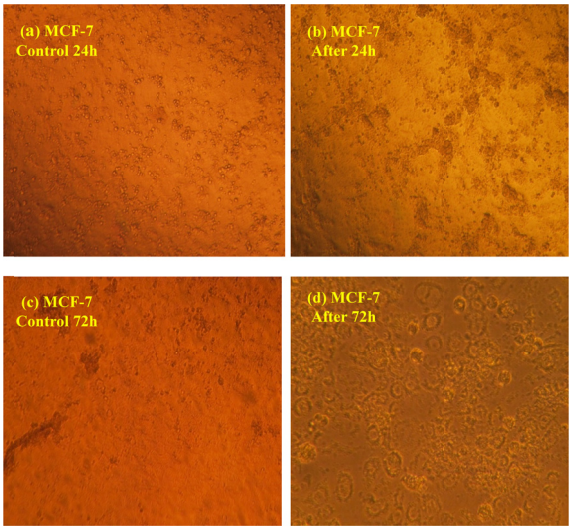

Figure 4. Morphological analysis of MCF-7 cells: **(a, c)** control cells, **(b)** after 24 h exposure to simulated microgravity, **(d)** after 72 h exposure to simulated microgravity.

MCF-7 cells Morphological Analysis

Since morphological analysis demonstrates the cell health, the MCF-7 cells were investigated for any modified morphology once subjected to microgravity. The MCF-7 cells grew as a monolayer once exposed to normal gravity (1g) after 24 h and 72 h (Fig. 4). As demonstrated in this figure, after 24 h exposure of cells in microgravity condition the morphology of cells slightly altered, and some cells became rounded. However, MCF-7 cells subjected to simulated microgravity for 72 h revealed significant morphological variations.

Growth Rates of MCF-7 cells

In the next step, we assessed whether microgravity simulation could change cell viability. MTT identifies cell death at later stages of apoptosis, wherein metabolization of tetrazolium salts is diminished. The MTT assay findings showed that microgravity simulation after 24 h or 72 h did not have a remarkable effect on the MCF-7 cells viability (Fig. 5a). The values of necrotic and apoptotic cells were measured through analyzing membrane integrity as well as externalization of phosphatidylserine (PS) through double staining with

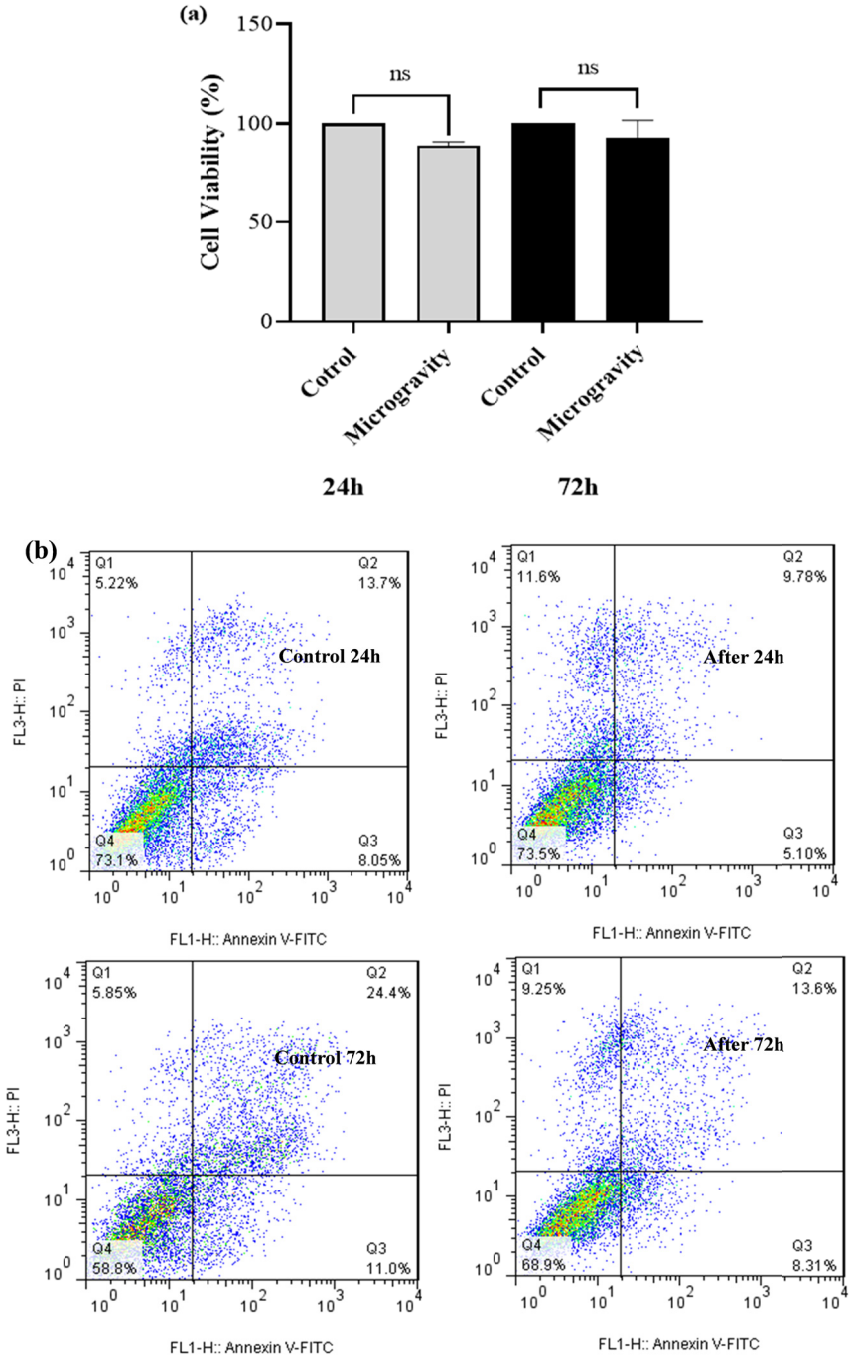

Figure 5. (a) MTT assay after 24 h and 72 h exposure to simulated microgravity. The data are obtained from the Means of three independent measurements ± SD (ns = non-significant). **(b)** Dot plots of PI against Annexin V staining followed via flow cytometry analyses.

PI and Annexin V through flow cytometry. According to Figure 5b, after 24 h and 72 h of simulated microgravity, the population of viable cells did not display remarkable variations compared to control cells.

Discussion

Humans on Earth have protected from dangerous elements that originated from deep space. Conversely, during space exploration, cosmonauts are subjected to

microgravity. Currently, various space exploration investigations have been done to understand the variations in biological function and structure of living organisms in microgravity condition. Presently, it has been discovered that microgravity directly can alter the morphology and function of biosystems [4, 5, 18]. In this work, at first, the real-time RT qPCR method was utilized to measure the simulated microgravity effect on the expression of the claudin-1 and claudin-3 genes. Compared to the control group, after 24 h of simulated microgravity, the expression of claudin-1 did not change, and the claudin-3 expression diminished very slightly ($P<0.05$). After 72 h of simulated microgravity, the claudin-3 and claudin-1 expression increased significantly ($P<0.01$). Thus, based on our data claudin-1 was up-regulated in MCF-7 cells. The claudin-1 subcellular localization has been demonstrated to be disrupted in invasive breast tumors [19]. Hoevel *et al.* showed that claudin-1 alone could be adequate to exert a tight junction-mediated gate function in metastatic cancer cells [19]. Achari *et al.* found that MCF-7 cells as estrogen receptor-positive breast cancer cells did not depend on claudin-1 for survival. They also demonstrated that reduction of claudin-1 can induce apoptosis and also lead to modifications in the JNK and ERK1/2 pathways [20]. Zhou *et al.* revealed that overexpression of claudin-1 in the MCF-7 cell line could increase migration as well as could enhance cell numbers compared to control cells [21].

Based on our data claudin-3 was up-regulated. Todd *et al*. demonstrated a noticeable overexpression of claudin-3 protein in MCF-7 breast adenocarcinoma [22]. Consequently, the overexpression of claudin-3 is probably involved in tight junctions' disruption in this breast tumor, therefore facilitating metastasis [22].

Under microgravity condition, the morphology of cells changed and became larger at day 3 compared to earlier days of growth, i.e., MCF-7 cells after 72 h exposure to microgravity simulation comprised rounded cells, which were grouped and linked to each other making multicellular spheroids (MCS) floating in the supernatant. Our observation is coincident with those published by Kopp *et al.* [23] who explored MCF-7 cells for several days under microgravity condition. The authors detected the first MCS after 24 h of exposure and they reported that the MCS continued to expand in complexity and number until 120 h. It is important to mention that multicellular architecture is one of the defining breast cancer properties and unfortunately most *in vitro* tumor models fail to renovate tumor architecture. However, multicellular architecture is well-known to drive the malignant tumor progression through cell-matrix- and cell-cell-contacts, diminished tensional homeostasis, as well as forced depolarization [6]. Masiello *et. al.* remarked that simulated microgravity causes distinct phenotypic switch on MDA-MB-231 breast cancer cells [10]. Our observation is also coincident with other observations [7, 8]. According to the literature, any alterations in the cells shape are correlated with variation in cytoskeleton architecture. The balance between the tensional forces and cytoskeleton architecture controls several complex cell functions, for example, proliferation and apoptosis directly [10]. As revealed earlier, gravity, as an external field, significantly influences the bio-system in choosing one out of various other structures. Even though, in microgravity condition, the bio-system can access various attractor states without limitations, and recover new phenotypes or configuration states [10, 24]. So, it seems that when MCF-7 cells were subjected to microgravity could obtain different phenotype.

The results of the MTT assay demonstrated that microgravity simulation after 24 h or 72 h did not have a remarkable effect on the MCF-7 cells viability (Fig. 5a). Recently, Ebnerasuly *et al.* [25] have investigated the effect of microgravity on adipose-derived stem cells. Interestingly, the viability of adipose-derived stem cells didn't show significant differences after 7 days of simulated microgravity condition in comparison with normal gravity condition. Our observation is also coincident with the previous study which pointed out that under simulated microgravity condition the viability of MDA-MB-231 cells didn't show significant differences [7].

The flow cytometry analysis also showed that after 24 h and 72 h of simulated microgravity the population of viable cells did not display remarkable variations compared to control cells. Chen *et al.* [9] examined MDA-MB-231 cells as well as MCF-7 cells for 120 h on the RCCS (Rotary Cell Culture System). They indicated that simulated microgravity did not alter the overall growth rate of these breast adenocarcinomas. Previous research also showed distinct behaviors of the malignant MCF-7 cells: an increment in apoptosis after 24 h [23], and no apparent variation in apoptosis-related proteins after 48 h [26]. Masiello *et al.* [10] explained that the apoptotic progression was increased in non-adherent MDA-MB-231 breast cancer cells after 24 h and 72 h simulated microgravity, in contrast to adherent MDA-MB-231 cells which exhibited slight alterations in apoptosis.

Collectively, based on our results, even though the relative mechanisms require to be investigated further, after 72 h of simulated microgravity, the claudin-1 as well as claudin-3 expression increased significantly. Subsequently, the overexpression of claudin-3 and

claudin-1 could induce tight junctions' disruption, increase migration as well as enhance cell numbers compared to control cells [6]. Furthermore, MCF-7 cells after 72 h exposure to microgravity simulation comprised rounded cells, which were linked and grouped to each other forming multicellular spheroids. However, microgravity simulation after 24 h or 72 h did not have a remarkable effect on the viability of MCF-7 cells. Nowadays, researchers have discovered some evidence that indicates microgravity could initiate some types of cancer like breast, leukemia, ovarian, neck lung, liver, and head cancers. For example, epidemiological investigations have displayed an enhanced breast cancer incidence in female commercial flight attendants [6]. Thus, sending patients into space could not be a remedy for cancer. Conversely, the aggressiveness of some cancer cells appears to be diminished *in vitro* after simulated microgravity exposure [6]. To describe this disagreement, it is worth revealing that different bio-systems were explored *in vitro* do not always reveal the complex situation *in vivo* which also comprises cell-cell interactions as well as the crosstalk between various body systems in space. However, microgravity research can offer a distinctive *in vitro* tool to explore biomechanical impacts in the biology of cancer. For example, Hekmat *et al.* [7] proved that 48 h simulated microgravity can improve the antiproliferative effect of the sterilized TiO_2 nanoparticles on MDA-MB-231 breast cells and as a result cell viability reduced markedly. Consequently, it seems that microgravity could not directly be a decision for tumor treatment, however, supports tumor investigation in two different ways: First, microgravity research has unraveled some significantly fascinating aspects of the biology of cancer; second, it offers a trustable *in vitro* 3D cancer model for preclinical anti-tumor drug discovery. Although, the finding from this study can be utilized to improve survivability and quality of life for cancer patients. However, lots of investigations focused on progression and cancer development in space as well as microgravity simulation are still required.

Conclusion

In this study, after 72 h of simulated microgravity, the claudin-1 and claudin-3 expression increased significantly. Furthermore, MCF-7 cells after 72 h exposure to microgravity simulation comprised rounded cells, which were linked and grouped to each other making multicellular spheroids. However, microgravity simulation after 24 h or 72 h did not have a remarkable effect on the viability of MCF-7 cells. Consequently, simulated microgravity could not be a cure for patients with breast cancer. However, microgravity research can offer a distinctive *in vitro* tool to explore biomechanical impacts in the biology of cancer. Our observation can open fascinating research lines in astrobiology, biophysics, and cancer biology. The finding from this study can be utilized to improve quality of life and survivability for cancer patients.

Acknowledgments

The authors would like to acknowledge supervisors of genetics lab in Zakariya Razi Laboratory Complex of the Science and Research Branch, Islamic Azad University for their enabling help and continuous support.

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