Culture of human cells in experimental units for spaceflight impacts on their behavior

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Impact statement

Cell cultures represent valuable preclinical models to decipher pathogenic circuitries. This is true also for biomedical research in space. A lot has been learnt about cell adaptation and reaction from the experiments performed on many different cell types flown to space. Obviously, cell culture in space has to meet specific requirements for the safety of the crew and to comply with the unique environmental challenges. For these reasons, specific devices for cell culture in space have been developed. It is important to clarify whether these alternative culture systems impact on cell performances to allow a correct interpretation of the data.

Abstract

Because space missions produce pathophysiological alterations such as cardiovascular disorders and bone demineralization which are very common on Earth, biomedical research in space is a frontier that holds important promises not only to counterbalance space-associated disorders in astronauts but also to ameliorate the health of Earth-bound population. Experiments in space are complex to design. Cells must be cultured in closed cell culture systems (from now defined experimental units (EUs)), which are biocompatible, functional, safe to minimize any potential hazard to the crew, and with a high degree of automation. Therefore, to perform experiments in orbit, it is relevant to know how closely culture in the EUs reflects cellular behavior under normal growth conditions. We compared the performances in these units of three different human cell types, which were recently space flown, i.e. bone mesenchymal stem cells, micro- and macrovascular endothelial cells.

Endothelial cells are only slightly and transiently affected by culture in the EUs, whereas these devices accelerate mesenchymal stem cell reprogramming toward osteogenic differentiation, in part by increasing the amounts of reactive oxygen species. We conclude that cell culture conditions in the EUs do not exactly mimic what happens in a culture dish and that more efforts are necessary to optimize these devices for biomedical experiments in space.

Keywords: Human bone mesenchymal stem cells, human endothelial cells, cell culture experimental unit, reactive oxygen species, osteogenic differentiation

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Introduction

Gravity has shaped life on earth. This concept became evident when the space race started in the 1950s. After hundreds of space missions it is clear that spaceflight and, in particular, long-duration missions activate adaptive responses that might result in potential hazard for the astronauts. Indeed, several pathophysiological alterations are reported that lead to cardiovascular deconditioning, spatial disorientation because of vestibular impairment, immune deficiency, muscle atrophy, and bone demineralization, among others.¹

We are interested in investigating the alterations of the bone and of the endothelium in microgravity.

As mentioned above, astronauts experience serious, weightlessness-induced bone loss and this is due to an unbalanced process of bone remodeling that involves osteoblasts, osteocytes, and osteoclasts.² The effects of microgravity on the cells of the bone have been studied in simulated

and real microgravity,³ but it is only recently that consideration has been given to the role of bone mesenchymal stem cells (bMSCs), which can differentiate into osteoblasts, chondrocytes, and adipocytes depending on the stimuli they receive.⁴ bMSCs also produce several factors that regulate bone homeostasis and hematopoietic progenitors.⁵ The effects of simulated microgravity on these cells are controversial.^{6–9} We have recently demonstrated that culture of bMSCs in the random positioning machine, a bioreactor which simulates microgravity, activates stress response, and accelerates the expression of several osteogenic genes.¹⁰ Experiments on bMSCs in real microgravity might help to better understand how these cells respond to mechanical unloading.

Also endothelial cells are particularly puzzling to study, not only because they are responsible for maintaining the integrity of the vessels, but also because they influence the neighboring tissues by secreting various molecules.¹¹

Endothelial cells are highly heterogeneous in structure and function, gene expression, and antigen composition.¹² Accordingly, heterogeneous responses are described in endothelial cells from different vascular beds and even in different sections of the same vascular bed. Simulated microgravity affects endothelial function and different responses to gravitational unloading have been described in micro- and macrovascular human endothelial cells.^{13–16} We have also shown that microvascular endothelial cells in simulated microgravity release proteins that affect osteoblast behavior in a co-culture system.¹⁷ Recently, both macrovascular¹⁸ and microvascular endothelial cells were flown to the International Space Station (ISS), which offers unique opportunities to study the effect of space on the cells.

For biological experiments in space, several requirements need to be met and specific closed cell culture systems (from now defined experimental units (EUs)), which must be safe, light, and functional, have been developed.¹⁹ For manned space flight there are rigorous safety requisites to minimize any potential hazard to the crew. In addition, because the daily schedule of astronauts is very tight, the EUs should possess a high degree of automation. Moreover, to reduce the cost of space missions, it is important that the EUs for cell culture are light. They must be biocompatible and provide the optimal conditions for the cells including the possibility of supplying fresh medium or specific reagents to collect and preserve the samples at the end of the experiment.

We have been involved in the preparation of some experiments on cells which were flown to the ISS. In particular, bMSCs, human umbilical vein endothelial cells (HUVECs), and human dermal microvascular endothelial

cells (HDMECs) were space flown in the EUs used in this study.

We investigated two main issues: (i) whether culture in the EU, which is a close system, induces oxidative stress. To find an answer, we cultured the cells in the EU and measured the production of reactive oxygen species (ROS); (ii) whether culture in the EU alters the behavior of the cells. In the case of bMSCs, we exposed the cells cultured in the EU to an osteogenic medium (OM) and evaluated the expression of *Runt-related transcription factor 2* (*RUNX2*) and *osterix* (*OSX*), crucial transcription factors in osteogenesis. For endothelial cells, we evaluated cell growth at 24 and 96 h after assembling the EUs.

Methods

Experimental Units (EUs)

The EUs, electromechanical devices for the autonomous execution of a scientific protocol in microgravity, were developed by Kayser Italia¹⁸ (Figure 1). Briefly, each EU is composed of a brick of biological compatible plastic (PEEK®) with a cell culture chamber, five cylindrical reservoirs to store media and chemicals, and a fluidic path for exchanging fluids. The culture chamber is designed to accommodate cells cultured in monolayer on 2.3 cm² Thermanox coverslips (Nunc, Roskilde, Denmark) with 1.3 mL of culture medium (CM). Each reservoir has a piston, which injects fresh fluids into the culture chamber so that the wasted fluids are collected in the empty cylinder. In these experiments, three reservoirs were filled as following: 1–2 contained phosphate buffered saline (PBS) to rapidly wash the monolayer, three contained various compounds, i.e. RNAlater to extract RNA, 4%

Figure 1 Experimental unit. EU and its components are shown before (a), during (b, c), and after (d) the assembling. (a) Gaskets (1) prevent contamination and liquid leakage; Thermanox coverslip (2) are used to culture the cells and are inserted into the Thermanox coverslip support (3) which is then assembled into the main body (6) and covered with the lateral cover (5); pistons (4) compress the fluids and valves (7) regulate the exchange of fluids.

paraformaldehyde (PFA) to fix the cells for microscopy, or PBS for determining ROS and counting the cells (Figure 2).

Cell culture

HDMECs were obtained from CDC (Atlanta) and grown in MCDB131 (Gibco, Thermo Fisher Scientific, Waltham, Massachusetts, USA) containing epidermal growth factor (10 ng/mL), hydrocortisone (1 mg/mL), 10% fetal bovine serum (FBS), and glutamine (2 mM).¹⁴ HUVECs (American Type Culture Collection) were cultured in M199 containing 10% FBS, glutamine (2 mM), endothelial cell growth factor (150 mg/mL), sodium pyruvate (1 mM), and heparin (5 units/mL).²⁰ Both HDMECs and HUVECs were cultured on gelatin-coated dishes (2% in water). bMSCs from adult human bone marrow of a male healthy volunteer were donated by Prof. Berti (Policlinico, Milan).¹⁰ These cells, which flew to the ISS in 2015, were cultured in Dulbecco's Modified Eagle's Medium with 1000 mg/L glucose and containing 10% FBS and 2 mM glutamine. In some experiments, bMSCs were cultured in the presence of the antioxidant *N*-acetylcysteine (NAC) (1 mM). All the cells were maintained at 37°C and 5% CO₂. All culture reagents were from Sigma-Aldrich, Saint Louis, Missouri, USA. To be cultured in the EUs, all these cells were seeded on Thermanox coverslips in their own CM supplemented with 12.5 mM HEPES to buffer culture media and kept overnight at 37°C before being loaded in the EUs. For endothelial cells, the coverslips were coated with 2% gelatin. Control cells on Thermanox were maintained in culture dishes with 1.3 mL of CM. All the cells were cultured at 37°C in the presence of HEPES. Endothelial cells were trypsinized, stained with trypan blue solution (0.4%), and the viable cells were counted using a cell counter just before

assembling the EUs (Time 0) and after 24 or 96 h. The experiments were performed at least three times. To study *in vitro* osteogenic differentiation of bMSCs, part of the samples were exposed to an OM containing 2×10^{-8} M 1 α ,25-dihydroxyvitamin D₃, 10 mM β -glycerolphosphate, and 0.05 mM ascorbic acid (Sigma-Aldrich).¹⁰

For morphology evaluation, hematoxylin-eosin staining was performed. Briefly, the cells were fixed with 4% paraformaldehyde (PFA) for 10 min and washed with PBS for three times. After adding hematoxylin for 3 min, the cells were dyed with eosin, dehydrated with gradient ethanol, soaked with xylene, and mounted with neutral balsam. Stained cells were photographed with a Zeiss Imager M1 microscope equipped with the AxioCam MRC5 camera using AxioVision 4.6 software (Carl Zeiss Microimaging GmbH, Gottingen, Germany).

Reactive oxygen species (ROS) production

For ROS quantification, the cells were detached from the Thermanox by trypsinization and resuspended in a 20 μ M 2'-7'-dichlorofluorescein diacetate (DCFH) solution. After 30 min of incubation in a 96-well black plate, the DCFH dye emission was monitored at 529 nm using Promega Glomax Multi Detection System. ROS production was normalized on the basis of cell number. The results are the mean of three independent experiments performed in triplicate. Data are shown as the fold increase in ROS levels of the samples compared to control cells cultured in dish.

Real-time polymerase chain reaction (PCR)

Total RNA from bMSCs was extracted by the PureLink RNA Mini kit (Ambion, Thermo Fisher Scientific). Single-stranded cDNA was synthesized from 0.2 μ g RNA in a 20 μ L

Figure 2 Schematic representation of fluid exchanges in the EUs. This protocol was used for all the cells tested.

final volume using High Capacity cDNA Reverse Transcription Kit, with RNase inhibitor (Applied Biosystems, Thermo Fisher Scientific) according to the manufacturer's instructions. Real-time PCR was performed in triplicate on the 7500 FAST Real-Time PCR System instrument using TaqMan Gene Expression Assays (Life Technologies, Carlsbad, California, USA): Hs00231692_m1 (*RUNX2*) and Hs01866874_s1 (*OSX*). The housekeeping gene *GAPDH* (Hs99999905_m1) was used as an internal reference gene. Relative changes in gene expression were analyzed by the $2^{-\Delta\Delta Ct}$ method.^{10,21}

Statistical analysis

Statistical significance was determined using Student's *t*-test and set as following: **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Results

Culture of bMSCs in the EUs

Confluent bMSCs on Thermanox were utilized. We measured ROS production by DCFH 24 and 96 h after

assembling the EUs and found a significant increase of ROS generation in bMSCs in the EUs (Figure 3(a)). To investigate whether this overproduction of ROS impacted on the response of the cells, we cultured bMSCs in the EUs in OM or CM. After 96 h the EUs were disassembled and cell morphology as well as the expression of osteogenic markers was investigated. As shown in Figure 3(b), the cells were elongated with a round nucleus and a prominent nucleolus. We did not detect any significant morphological difference in cells grown in CM versus OM in the culture dish. However, in cells cultured in the EUs and exposed to the OM, the cells are less than numerous in the controls and tend to align and organize in bundles.

Then we evaluated the expression of two markers of osteogenic differentiation, *RUNX2* and *OSX*, by real-time PCR after culturing bMSCs in the EU or in a dish in OM or CM for 96 h. Figure 3(c) shows that the induction of *RUNX2* and *OSX* is higher in cells in the EU than in the corresponding controls in a culture dish. To investigate whether this difference could be ascribed to the overproduction of ROS by bMSCs in the EU, we exposed the cells to

Figure 3 Effects of culture in the EUs on bMSCs. (a) ROS generation was measured by DCFH 24 and 96 h after assembling the EUs. Data are shown as the fold increase in ROS levels of the samples compared to 24 h control cells cultured in dish. Each bar is the mean of three separate experiments $\pm$ standard deviation. (b) Microphotographs of cells stained with hematoxylin-eosin were taken 96 h after culture in the EU or in control dish. (c) RNAs were extracted and a real-time PCR was performed using primers designed on *RUNX2* and *OSX* sequence.

the synthetic antioxidant NAC (1 mM) for the duration of the experiment. We found that NAC decreased the expression of *RUNX2* and *OSX* in bMSCs cultured in the EU in OM to levels comparable with the controls cultured in a dish (Figure 3(c)), thus indicating that the increased production of ROS is involved in the accelerated expression of osteogenic markers.

Culture of HDMECs in the EUs

10^4 HDMECs were seeded on Thermanox coated with 2% gelatin. Twenty-four and 96 h after culture in the EUs, we measured ROS production by DCFH and did not detect any significant difference (Figure 4(a)). It is noteworthy that ROS production dropped after 96 h when the cells reach confluence, indicating that ROS production is higher in sparse versus confluent cells. Indeed, while after 24 h proliferation in the EUs was retarded, no significant differences of cell number were observed after 96 h (Figure 4(b)).

Culture of HUVECs in the EUs

A total of 5×10^3 HUVECs were seeded on gelatin-coated Thermanox. We measured ROS production by DCFH 24

and 96 h after assembling the EUs. As shown in Figure 5(a), after 24 h we found decreased amounts of ROS in cells in the EUs, while no differences were detected between the two experimental conditions after 96 h. It should be pointed out that the reduced generation of ROS after 96 h is a common feature in HUVECs and HDMECs (Figure 4(a)). When we counted the cells, we found more cells in the EUs after 24 h, while after 96 h the number of cells was comparable in the EUs and in the controls (Figure 5(b)).

Discussion

Our data show that when cultured in the EUs the behavior of three different cell types is different from controls grown under normal condition. It is noteworthy that HUVECs, HDMECs, and bMSCs were all cultured in these EUs to be flown to space for experiments onboard the ISS. For HUVECs the data of the experiment onboard the ISS have been published,¹⁸ while the postflight analyses on HDMECs and bMSCs are in progress.

bMSCs are a rare population in bone marrow and show a broad spectrum of differentiation potential.⁴ In response to osteogenic stimuli, they activate a genetic program leading

Figure 4 Effects of culture in the EUs on HDMECs. (a) ROS generation was measured 24 and 96 h after assembling the EUs. Data are shown as the fold increase in ROS levels of EU cultured cells compared to controls cultured in dish. Each bar is the mean of three separate experiments $\pm$ standard deviation. (b) After 24 and 96 h, viable cells were counted.

Figure 5 Effects of culture in the EUs on HUVECs. (a) ROS generation was measured 24 and 96 h after assembling the EUs. Data are shown as the fold increase in ROS levels of EU cultured cells compared to controls cultured in dish. Each bar is the mean of three separate experiments $\pm$ standard deviation. (b) After 24 and 96 h, viable cells were counted.

to differentiation into osteoblasts, the cells in charge of bone formation. Since no ossification occurs in *RUNX2* knockout mice,²² *RUNX2* is considered the master regulator of osteogenesis. *OSX* is necessary to promote the early stages of osteogenesis, but it is not sufficient to reach complete differentiation.²² Here we show that culture in the EUs accelerates bMSCs genetic reprogramming by overexpressing *RUNX2* and *OSX* in response to OM. This finding might be of interest not only for scientists preparing experiments for space biology but also for professionals involved in the growing field of regenerative medicine in orthopedics, with the aim of replacing or repairing diseased or injured skeletal tissue. We also found an increased generation (about three-fold induction) of ROS in the EUs. In general, while an excess of ROS is cytotoxic, an adequate amount of ROS is required to maintain cell proliferation, self-renewal, and regulation of differentiation.²³ In bMSCs and in preosteoblasts, the generation of ROS is needed for osteogenic differentiation.^{24,25} Indeed, since the antioxidant NAC reduces the expression of *RUNX2* and *OSX* in bMSCs exposed to OM in the EUs, we propose that the induction of osteogenic differentiation markers is mediated, in part, by ROS. It should also be noted that increased amounts of ROS might activate an adaptive response that enhances differentiation. Indeed, some stress proteins are important in regulating bMSCs performances. In particular, HSP70 induces the expression of *RUNX2* and *OSX*, thus promoting osteogenesis.²⁶

HUVECs are widely used as a model of macrovascular endothelial cells. These cells were studied in simulated microgravity generated by the random positioning machine and the rotating wall vessels.^{13,16,27} In 2010, these cells were flown to the ISS after being assembled into the EUs used in this study.¹⁸ Space modulates the expression of more than 1000 genes.¹⁸ Here we show that cell number is increased in the EUs after 24 h, suggesting that the microenvironment generated in the closed system of the EU favors HUVECs growth. At 96 h both EU cultured and control cells cultured in dish reach confluence. It is interesting to note that ROS production is lower in HUVECs in the EUs after 24 h. In addition, ROS are markedly decreased after 96 h in HUVECs in the EUs and in the dish, when the cells are confluent. These data are in agreement with the findings that cell number is inversely related to the production of ROS in HUVECs (data not shown). In space-flown HUVECs, on the basis of gene expression studies, an increase of oxidative stress has been postulated.¹⁸ The results presented here suggest that oxidative stress is not generated by culture in the EUs, but it is possible that space environmental factors, such as vibrations, microgravity, and radiations, might induce the production of ROS.

HDMECs in the EUs behave differently from HUVECs. We found an initial and transient growth retardation, while after four days the cells in the EUs and their controls in the dish are confluent. We hypothesize that HDMECs might require some adaptive response early after culturing in the EUs, but they rapidly rescue their normal proliferation rate and reach confluence within four days like the controls. Differently from HUVECs and bMSCs, these cells do not alter ROS production in the EUs. The differences between

HUVECs and HDMECs might be ascribed to the fact that endothelial cells from different vascular beds are heterogeneous in terms of function, structure, and responses to *in vitro* stimulation. Accordingly, we demonstrated the different response to simulated microgravity of these cells.^{14,16} There is one interesting similarity in the behavior of these cells, i.e. the production of ROS dramatically drops when HUVECs and HDMECs are confluent, thus indicating an inverse correlation between cell number and ROS generation.

We conclude that culturing cells in the EUs influences cell behavior and this is particularly evident in bMSCs, while both micro- and macrovascular endothelial cells seem to rapidly adapt to culture in these devices. To this purpose, it is well known that endothelial cells show a remarkable capacity to suit local requirements and to comply with different humoral, neural, and mechanical stimuli.²⁸

Several spaceflight hardware are available and, when planning experiments with cells in space, it would be interesting to evaluate whether and how these different devices affect cell performances. This will help to design proper protocols to ensure the optimization of the experimental conditions so that culture in the EU can reflect as closely as possible what happens in a culture dish. This approach will facilitate the interpretation of the results and the comparison with data obtained on earth under normal culture conditions.

Authors' contribution: AC, CM, JAMM, and SC conceived and designed the experiments. SC and AC performed the following experiments: cell culture, ROS quantification, real-time PCR; CM performed the hematoxylin-eosin staining and took the microphotographs of the cells; AC, CM, JAMM, and SC analyzed the data. JAMM wrote the paper. All authors reviewed the manuscript.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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