Journal of Bioscience and Bioengineering
VOL. 113 No. 2, 258–261, 2012

ELSEVIER

www.elsevier.com/locate/jbiosc

NOTE

Hypergravity effects on myoblast proliferation and differentiation

Gianni Ciofani,[1,*] Leonardo Ricotti,[2] Jacopo Rigosa,[2] Arianna Menciassi,[2] Virgilio Mattoli,[1] and Monica Monici [3]

Italian Institute of Technology, Center of MicroBioRobotics c/o Scuola Superiore Sant'Anna, Viale Rinaldo Piaggio 34, 56025 Pontedera (Pisa), Italy,[1] The BioRobotics Institute, Scuola Superiore Sant'Anna, Viale Rinaldo Piaggio 34, 56025 Pontedera (Pisa), Italy,[2] and ASAcampus Joint Laboratory, ASA Research Division, Dept. Clinical Physiopathology, University of Florence, Viale Pieraccini 6, 50139 Florence, Italy[3]

Received 13 September 2011; accepted 30 September 2011
Available online 4 November 2011

This study aimed at the investigation of behavior of myoblasts in conditions of altered gravity. C2C12 cells underwent stimulations by different hypergravity intensities (2 h at 5*g*, 10*g*, and 20*g*) in the Large Diameter Centrifuge of the European Space Agency (ESA), highlighting positive effects on both proliferation and differentiation.

[**Keywords:** Hypergravity; Large diameter centrifuge; C2C12 cells; Cell proliferation; Cell differentiation]

Skeletal muscle undergoes adaptive changes in both fiber size and contractile phenotype in response to conditions of altered loading states (e.g., microgravity or hypergravity). It is well known in fact that long term exposure to microgravity can lead transient postural-motor deficits after re-exposure to gravitation (1). In particular, spaceflights have been shown to cause atrophy and reduction in force and power of skeletal muscle (2).

Therefore, while muscle behavior in microgravity has been the subject of many studies, a few papers can be found in the literature that address the same biological problem in hypergravity conditions. Oguro et al. (3) showed that the expression of HSP47, a collagen-specific molecular chaperone, responds to gravitational changes, including microgravity and hypergravity *in vitro* and *in vivo*. Another study by Hirasaka et al. (4) investigated hypergravity-induced protein ubiquitination of L6 muscle cells, demonstrating that ubiquitination was significantly induced by a 1 h treatment at 100*g*.

Starting from these interesting results, our goal was an evaluation of the C2C12 cell parameters affected by a medium term (2 h) exposure to different levels of hypergravity.

The collected data on proliferation and differentiation processes of myoblasts during exposure to different levels of hypergravity could in fact not only provide a significant contribution in understanding muscle behavior at altered gravity forces (5), but also suggest appropriate culture stimuli for tissue engineering applications (6).

Experiments were performed in the Large Diameter Centrifuge (LDC) system of the European Space Agency (ESA, Noordwijk, The Netherlands) in the framework of the "Spin Your Thesis!" program. The LDC system is used for experimental hypergravity setups, and comprehends a large rotating arm where a swing gondola is attached at the extremity. The rotational movement of the arm and gondola creates an artificial acceleration field at the equipment positioned inside the gondola: it suffers an artificial acceleration a_e (experimental acceleration) as a result of the earth gravitational acceleration and the centripetal acceleration developed by the rotating arm speed.

The set-up of our experiments was extremely simplified in order to perfectly fit the gondola requirements. Over a metal rack fixed at the bottom of the gondola, a Delrin structure hosted the supports for fixing 30 sealed cylindrical vials, completely filled with agar gel in order to prevent shear stress phenomena. Glass coverslips (12 mm in diameter) with the cell cultures were located on the top of the agar gel inside the tubes, and covered with 1 ml of culture medium.

C2C12 mouse myoblasts (CRL-1772, ATCC, passage <15) were cultured in proliferation medium, consisting in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. During culture, cells were maintained at 37°C in a saturated humidity atmosphere containing 95% air and 5% CO_2. This cell line, widely used as muscle cell model (7), can rapidly differentiate in appropriated culture conditions, also forming contractile myotubes that produce characteristic muscle proteins.

For proliferation assessment, cells were cultured on coverslips at a density of 10,000 cells/cm^2. After 24 h from seeding, samples underwent stimulation (2 h at 5*g*, 10*g* and 20*g*) in the LDC, while control cultures were performed at 1*g* at the same conditions. DNA and protein synthesis evaluation was performed at 24 h after starting of the hypergravity stimulation.

The ds-DNA content has been assessed because it is directly proportional to the sample cellularity, and, therefore, provides a clear indication on cell proliferation (8). Ability of protein synthesis has been investigated as an index of cell metabolic activity, thus providing information about the cell metabolism. ds-DNA content in cell lysates has been measured by using the PicoGreen kit following manufacturer's

* Corresponding author: Tel.: +39 050883019, +39 050883468 (Lab.); fax +39 050883497.
E-mail addresses: g.ciofani@sssup.it, gianni.ciofani@iit.it (G. Ciofani).

1389-1723/$ - see front matter © 2011, The Society for Biotechnology, Japan. All rights reserved.
doi:10.1016/j.jbiosc.2011.09.025

instruction (Molecular Probes) (9) and measuring the fluorescence intensity on a plate reader (Victor3, PerkinElmer) using an excitation wavelength of 485 nm and an emission wavelength of 535 nm. Total protein concentration has been determined by the bicinchoninic acid (BCA) method (Pierce) (10) following the microplate procedure. Absorbance was read at 562 nm on a plate reader (Victor3, PerkinElmer).

Actin staining was performed on treated samples immediately fixed after the hypergravity exposure. The organization of actin microfilament network in treated and non-treated cells was evaluated by f-actin staining with FITC-phalloidin, according to the following procedure. After hypergravity exposure, cells were rinsed with PBS 1×, then fixed using paraphormaldeyde (Sigma) 4% in PBS for 15 min. Thereafter, cells were rinsed with PBS three times (5 min each) and treated with Triton X-100 (Sigma), 0.1% in PBS for 15 min, for membrane permeabilization. Saturation was allowed for 10 min, using 0.1% porcine gelatin (Sigma) in PBS, then incubation with a 100 μM solution of FITC-phalloidin (Invitrogen) and 1 μM DAPI for nucleus blue counterstaining was performed for 30 min. After incubation, samples were rinsed in PBS High-Salt (0.45 M NaCl in PBS) for 1 min, then rinsed with PBS three times (5 min each) before observation with an inverted fluorescent microscope (Eclipse Ti, Nikon) equipped with a cooled CCD camera (DS-5MC USB2, Nikon) and with NIS Elements imaging software.

High magnification fluorescent images of several cells (>10 per sample, $n=3$) of the different cultures were processed with ImageJ as follows: the green channel component of each image was converted to a binary image and, by using a polygonal shape tool, the single cells were selected and the corresponding average fluorescence signal intensity in the green channel was measured, thus obtaining an index related to the f-actin amount in the considered cells.

Differentiation of C2C12 myoblasts in myotubes was induced by switching, at cell confluence on the coverslips, the culture medium from expansion to differentiation medium, the latter containing DMEM supplemented with 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 1% insulin–transferrin–sodium selenite (ITS, Sigma), and 1% FBS. After 24 h since medium switching, samples underwent stimulation (2 h at 5*g*, 10*g* and 20*g*) in the LDC, while control cultures were performed at 1*g* at the same conditions.

After 24 h from the hypergravity exposure, the differentiation process was evaluated through fluorescent staining of myogenin and myosin. Samples were rinsed with PBS and fixed in paraformaldehyde (4% in PBS) for 15 min. After 3 rinses with PBS (5 min each), they were incubated with sodium borohydryde (Sigma) (1 mg/ml in PBS) for 10 min to reduce or suppress autofluorescence and aspecific fluorescence. Cellular membranes were then permeabilized with 0.1% Triton X-100 in PBS for 15 min. Antibody aspecific binding sites were saturated with 10% goat serum in PBS for 1 h, and subsequently a primary antibody (rabbit polyclonal IgG, anti-myogenin or anti-myosin, Santa Cruz) diluted 1:75 in 10% goat serum was added. After 30 min of incubation at 37°C, the samples were rinsed 5 times (3 min each) with 10% goat serum; then the staining solution, composed by a secondary antibody (fluorescent goat anti-rabbit IgG, Invitrogen) diluted 1:250 in 10% goat serum and by 1 μM DAPI, was added. After 30 min of incubation at room temperature, the samples were rinsed with 0.45 M NaCl in PBS for 1 min to remove weakly bound antibodies. After 3 rinses in PBS (5 min each), the samples were observed with an inverted fluorescent microscope and 5 pictures for each sample were taken at 10× magnification on random fields. ImageJ software was subsequently used for all image analyses: number of myogenin-positive nuclei/total number of nuclei ratio, and myotube length and width, measuring at least 20 myotubes for each picture.

Analysis of the data was performed by analysis of variance (ANOVA) followed by Student's *t*-test to test for significance, which was set at 5%. Experiments were performed in triplicate if not otherwise specified. Results are presented as mean value ± standard error of the mean (SEM).

Hypergravity positively affected cell proliferation, as outlined by data shown in Fig. 1a. Initial DNA concentration (at the time of seeding) for all the samples was 0.10 ± 0.05 μg/ml. Cultures that underwent 10*g* and 20*g* stimuli exhibited a significant increment (about 1.5 and 3.5 times, respectively) of DNA content (and therefore of cell numbers) with respect to the controls (1*g*) and to the 5*g* experiment ($p<0.05$). Also the protein synthesis capability was positively affected by the stimulation; even if only at 20*g* we appreciated a significant increment of the total protein concentration in the samples (Fig. 1b).

Actin is the monomeric subunit of two types of filaments: microfilaments, one of the three major components of the cytoskeleton, and thin filaments, part of the contractile apparatus in muscle cells (11). Microfilaments (12) are most concentrated just beneath the cell membrane, and are responsible for resisting tension and maintaining cellular shape, forming cytoplasmatic phylopodia and lamellipodia. It has been demonstrated that hypergravity strongly affects the reorganization of f-actin filaments (13). In our case, cytoskeleton conformation was analyzed as shown in Fig. 2a–d (images of 1*g* (control), 5*g*, 10*g* and 20*g* treated cultures, respectively): an increment of f-actin filament thickness (red arrows) is evident at higher *g* values, compatible with a rearrangement of the cell structures already described in the literature (14). The increment of f-actin formation clearly emerges from the quantitative evaluation of the green fluorescence reported in Fig. 2e. In the 5*g* and 10*g* samples, the fluorescence signal level is almost 45% greater than in the 1*g* control culture, with no significant differences between the 5*g* and 10*g* samples ($p>0.05$). Higher values are measured in the 20*g* sample, with a fluorescence signal that is almost double respect to the 1*g* control and almost 35% greater than that measured for the 5*g* and 10*g* samples.

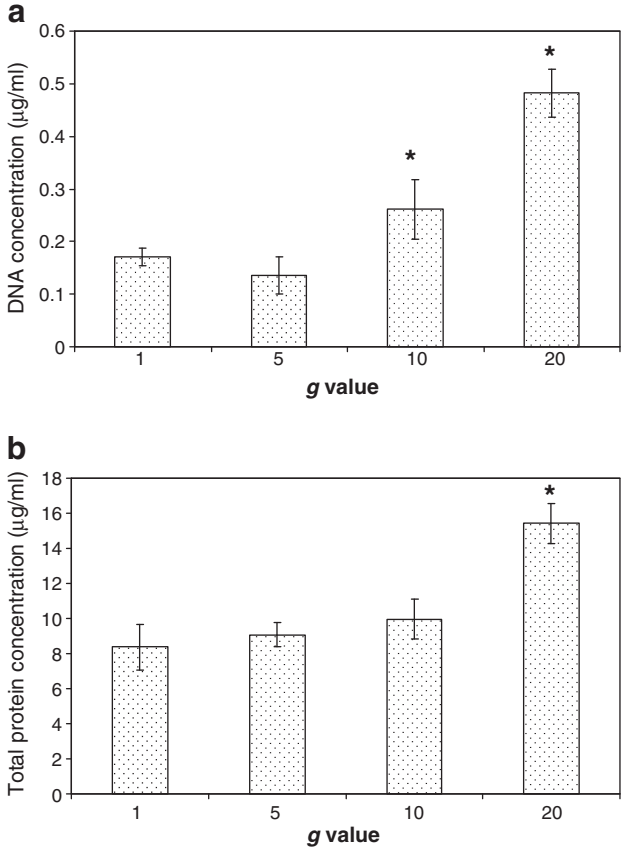

FIG. 1. DNA (a) and protein (b) content in cell cultures stimulated with different *g* values ($n=3$; *, $p<0.05$).