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"In vivo" physiological experiments in simulated microgravity conditions on rat bone marrow cells mineralization

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Abstract

The main goal of this research is to further contribute to the validation of the clinostat Random Positioning Machine (RPM) for "in vivo" experiments by studying bone marrow cells mineralization in the rat. These cells were obtained from the bone shafts of the femora and cultured for 13 days. The production of calcified matrix was analyzed by Alizarin Red staining demonstrating that calcium deposition was significantly decreased in RPM samples. Moreover, we demonstrated what Alkaline phosphatase (ALP) activity, a marker of osteoblastic phenotype, is down regulate by simulated microgravity. Also in vitro experiments in coltured osteoblasts (first subcolture) exposed to RPM for 9 days confirm a strongly decreased mineralization and differentiation.

Introduction

Space flight determines many serious physiological changes which include loss of muscle mass and bone, space motion sickness, anemia and a reduced immune response. The limited access to experimentation in real microgravity in space strongly suggests to use ground-based facilities for long or short duration exposure of humans (e.g. bed rest) or small-sized mammals to simulated low gravity conditions. The Random Positioning Machine (RPM) has been used so far as a suitable facility for *in vitro* studies. In our previous research, the first concerning rats exposed to simulated microgravity conditions in RPM, we observed that response to carrageenin or PGE_2-induced paw edema, thermal hyperalgesia and intestinal transit were significantly reduced (Peana et al., 2004). Moreover, the manufacture of Mouse Habitat on RPM (MHOR), a system which can permit to house 1 or 2 mice or rats on a RPM for a time interval up to 20 days with a minimum involved of personel, is currently in progress.

Several investigations performed in low gravity demostrated that microgravity causes decreased bone formation in combination with increased resorption leading to calcium and mineralized bone loss (Marie *et al.*, 2000). The progressive loss of calcium and bone may be the most critical biomedical obstacle that astronauts in extended duration space flights (e.g. Mars mission) will encounter . The main goal of this research was to further contribute to the validation of the RPM as facility for *in vivo* studies; therefore bone marrow cells mineralization in young male rats exposed to simulated microgravity has been investigated.

Methods

Young male rats were kept in a perspex semicylinder fixed to the inner frame center of clinostat (RPM, Fokker Space), within a second rotating frame for 72 hours. Rotation of each frame is random, autonomous and regulated by computer software with separate motors in order to obtain at the center of the inner frame low gravity simulated conditions. The first ground control group (Control RPM, CR) was kept individually in a perspex semicylinder on the basement of RPM and exposed to the same experimental conditions, while a second ground control group (Control Cage, CC) was kept in standard cage. All animals were given standard laboratory diet and water available *ad libitum* three times per day. The present study was carried out in accordance with the Italian low, which allows experiments on laboratory animals only after submission of a research project to the competent authorities, and according to the "Principles of laboratory animal care".

Bone marrow cells were obtained from the femora bone shafts and cultured for 13 days in α-Minimum Essential Medium supplemented with 15% fetal bovine serum, 10 mM HEPES, 10 mM β-glycerophosphate, 0,2 mM L-ascorbic acid 2-phosphate magnesium salt. Production of calcified matrix was detected by Alizarin Red staining for calcium deposition. Cells were fixed in 10% neutral-buffered formalin and stained with 40 mM Alizarin Red (pH 4.2). The precipitate was solubilized using 10% (w/v) cetylpyridinium chloride (CPC) in 10 mM sodium phosphate and the ARS concentration was determined by absorbance

measurement at 550 nm using an ARS standard curve in the same solution. Values were normalized to the total proteins.
Moreover, alkaline phosphatase (ALP) activity, a marker of osteoblastic phenotype, was determined using a fluorimetric assay (excitation at 360 nm and detection at 450 nm). Enzimatic activity was normalized to the total protein concentration.
Analysis of cell area and F-actin network was performed by immunofluorescence technique after culturing cells in a gas-permeable cell culture disks (OptiCell).
F-actin staining: cells were fixed with 4% paraformaldehyde, labelled with phalloidin-TRITC-conjugated and F-actin structures were detected by fluorescence-inverted microscope (20X magnification).
Cell area distribution: cells were fixed with 4% paraformaldehyde, labelled with primary monoclonal anti β-tubulin followed by a secondary FITC-conjugated antibody and observed with fluorescence-inverted microscope at 20X magnification. Cell area was calculated with the Image J software and the statistic analysis was performed by MYSTAT software.

Results and Discussion

Our results demostrated that simulated microgravity inhibited alkaline phosphatase activity and bone nodule formation in rat osteoblasts differentiated from bone marrow stromal cells. As shown in Fig. 1, Alkaline phosphatase appeared downregulated by simulated microgravity with a significant decrease by 54% (p< 0,05) compared to the cage control (GC) and by 35 % (p< 0,05) compared to the RPM control (CR). Also calcified matrix deposition was drammatically decreased by 95% (p<0,0001) in simulated microgravity conditions compared to the cage control (GC) and by 36 % (p< 0,001) compared to the RPM control (CR) (Fig. 2). Because the alkaline phosphatase is a well known marker and key regulator of osteoblast differentiation, our results suggest that microgravity inibits the differentiation of osteoblasts. Cell area distribution showed a significant decrease in low gravity conditions (Fig. 3). In both controls we observed that cells were distributed as a heterogeneous population including both small and big cells, whereas cells from rats exposed to simulated microgravity showed a homogeneous population with

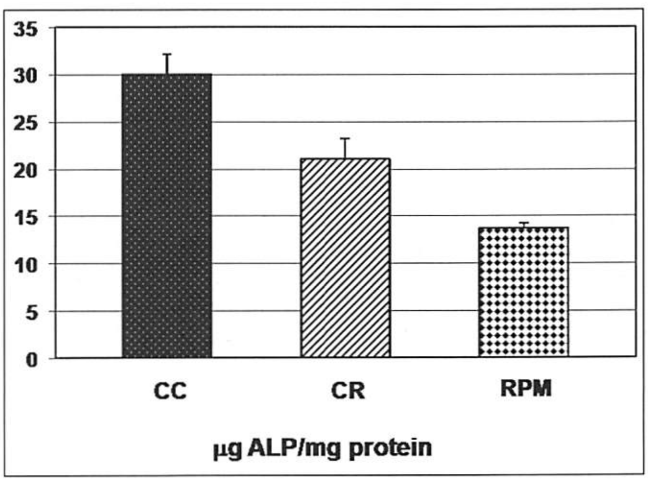

Fig. 1 - *Alkaline phosphatase activity (ALP) of osteoblasts differentiated from rat bone marrow.*

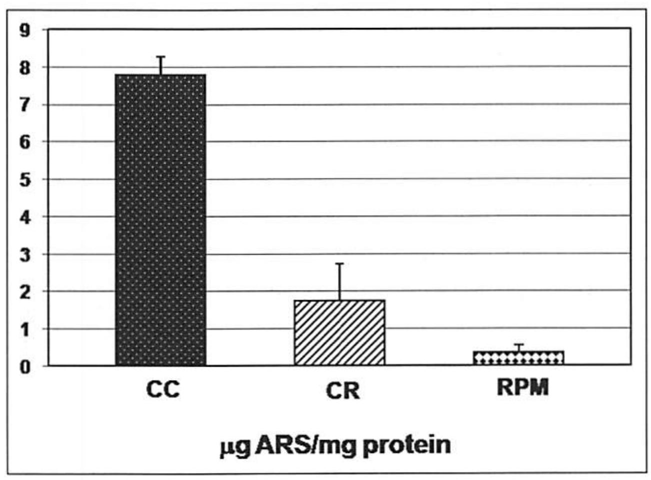

Fig. 2 - *Quantification of mineralization after Alizarin Red Staining (ARS) of osteoblasts differentiated from rat bone marrow.*

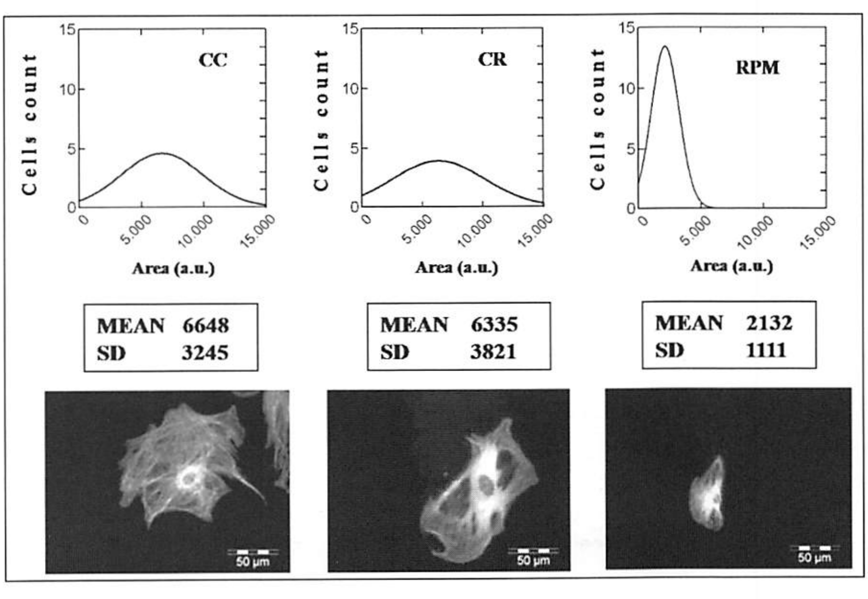

Fig. 3 - *Cell area distribution.*

significantly smaller cells. Moreover, the fluorescence staining of F-actin filaments showed a remarkable decrease in the filamentous biopolymer density (Fig.4). This result confirm the notable role of cytoskeleton in the mechanotrasduction of gravity signaling. Similar changes in the actin network, expecially a reduction of the stress fibers, were also observed in osteoblasts (Hughes-Fulford and Lewis, 1996), HUVEC cells (Buravkova and Romanov, 2001) and J111 monocytes (Meloni *et al.,* 2006) exposed to low g. In conclusion, our results can be considered a further contribute to the validation of the RPM and of the oncoming MHOR for *in vivo* physiological studies in simulated microgravity conditions.

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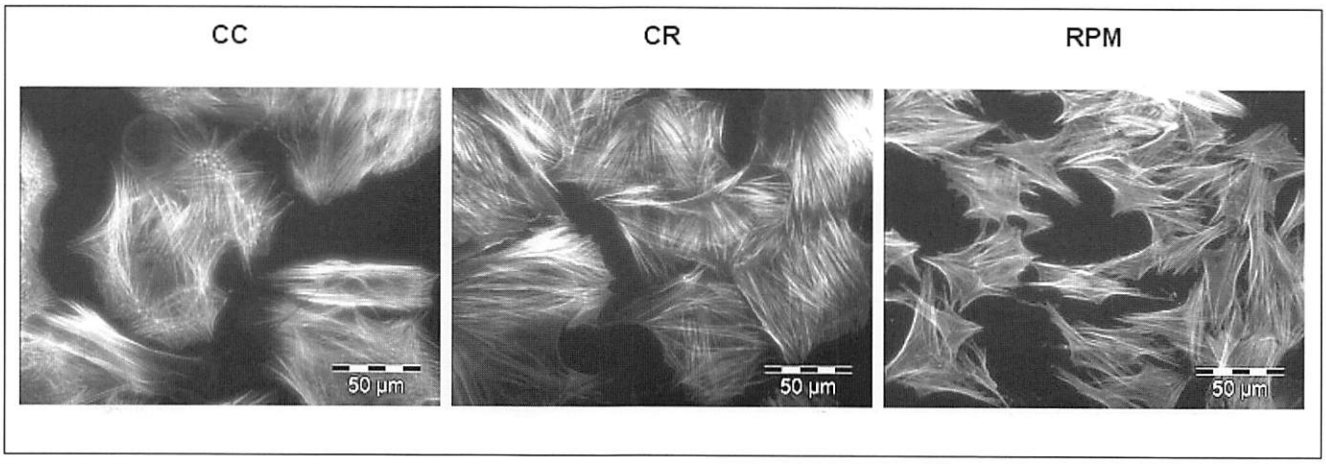

Fig. 4 - *The fluorescence staining of the actin network of osteoblasts differentiated from rat bone marrow.*