A MESOSCALE STUDY OF THE DEGRADATION OF BONE STRUCTURAL PROPERTIES IN MODELED MICROGRAVITY CONDITIONS

Francesca Cosmi [1)*], Nathalie Steimberg [2)], Giovanna Mazzoleni [2)]

[1)] Department of Engineering and Architecture, University of Trieste
via A. Valerio 10 - 34127 Trieste, Italy
[2)] Department of Clinical and Experimental Sciences, Faculty of Medicine and Surgery,
University of Brescia, viale Europa 11 - 25123 Brescia, Italy

*Corresponding author:
tel.: +39 (40) 558-3431; fax: +39 (40) 568-469
e-mail: cosmi@units.it

Abstract

One of the most important alterations that occur in man and experimental animals during spaceflight concerns the skeletal system, and entails important bone loss and degradation of mechanical properties. In the present work we investigate *ex vivo* the long-term effects of weightlessness (simulated microgravity) on bone tissue, by comparing the mesoscale structural properties of weight-bearing rat tibial epiphyseal cancellous structures of healthy animals (ground controls) with those of identical bone explants maintained *ex vivo* in the Rotary Cell Culture System (RCCS) bioreactor, used to model, on ground, microgravity conditions. Bone structures were reconstructed by synchrotron radiation micro-CT, morphometric analyses were performed, and the apparent elastic properties were computed by means of a numerical model based on the Cell Method. Two novel results were achieved in this study. First of all, the skeletal modifications found in bone explants after 3-4 weeks of culture in the RCCS bioreactor are in perfect agreement with those observed *in vivo* after a long-term spaceflight (*Mice Drawer System* mission, 2009), thus confirming the relevance of our model in reproducing the effects of microgravity on whole bone tissue. Secondly, but not less importantly, our study points out that the degradation in bone structural performance (apparent mechanical properties) must be considered in order to achieve an accurate representation of trabecular bone modifications not only in osteoporotic bone diseases, but also in the microgravity-induced bone alterations. In conclusion, our findings, by proving that the association of the RCCS bioreactor-based culture method, used to model microgravity conditions, with numerical simulations able to quantify bone quality, represents the first ground-based reliable model for investigating, *ex vivo,* some of the spaceflight effects on bone tissue, and open new perspectives to basic research and clinical applications.**1 Introduction**

Space travels into the terrestrial Orbit imply to be exposed to particular environmental conditions, where the gravitational force of the Earth is counterbalanced by centrifugal forces. This condition, called microgravity, is similar to free fall, and it is also known as near-

weightlessness. Experienced for long periods (weeks, months), microgravity induces various health problems in living beings (1), including serious skeletal alterations, which entail important bone loss and degradation of its mechanical properties. A decrease in gravitational loading induces, in effect, major bone changes, which comprise cancellous osteopenia, decreased bone formation, aberrant matrix ultrastructure and decreased mineralization (2 - 6), with a consequent increased risk of bone fracture. Although several hypotheses have been formulated during the past years, the mechanisms of spaceflight-induced bone modifications are still mostly unknown and need to be fully investigated, since skeletal fractures and increased osteoporosis still represent a serious medical scenario, which takes priority over other risks in the design of long-term space missions (e.g. Mars exploration) (7). The main problems in investigating the adaptation of the human skeleton to a microgravity environment derive, at present, from the limitations imposed by spaceflight on experimental procedures, and from the difficulty in having available (and reliable) *in vivo* experimental models, able to reproduce, on the ground, the environmental conditions typical of hypogravity. In addition, the current bone standards, mainly based upon osteoporosis diagnostic guidelines, are not acceptable for assessing the skeletal integrity of space travellers following prolonged spaceflight exposure (NASA Human Research Program, HRP, 2014) (8).

Long-term research studies under “real” microgravity conditions can be performed by the use of experimental platforms, such as the International Space Station (ISS), orbital capsules or sounding rockets (1). For animal studies, Mice Drawer System (MDS) - a particular device developed for the ISS as a facility to study long-time influence of various environmental conditions on the biology and behavior of mice during spaceflight (9) - has been recently employed for investigating the skeletal alterations occurred in mice after 3 months’ exposure to microgravity, and the results have been reported by the group of Cancedda and co-workers (10). While this study can undoubtedly provide invaluable insights

into zero-g physiology, the limited number of experimental mice (n=6) that can be housed aboard the ISS, and their low survival rate (50%), still represent critical aspects of this type of experiments. At present, spaceflight studies on living organisms have, in addition, major limitations: they are very expensive, must overcome enormous technical problems, and are extremely limited in size and frequency. Moreover, the results obtained are not uniform, due to the variable conditions under which they have been (or can be) undertaken. Therefore, various *in vivo* ground-based models of long-term effects of microgravity on bone tissue have been developed (see 11, and references therein).

To understand the mechanism(s) of microgravity effects on bone tissue means also clarifying the mechanism(s) of cell response to mechanical stimuli (mechanosensitivity of cells and mechanotransduction of signals), which, physiologically, regulate the complex homeostasis of bone remodeling (1). *In vitro* and *ex vivo* systems should then represent essential tools for investigating, at single cell level, the biological bases of bone response to spaceflight-produced weightlessness (12). Despite the great number of studies conducted in the past years by using microgravity-based culture systems, the results obtained are still inconclusive, and, often, conflicting and controversial (see, for example, 13 and 14). Among all the devices able to model microgravity condition on ground, the *Rotatory Cell Culture System* (RCCS™) bioreactor, fruit of N.A.S.A.'s technological research in U.S.A., presents the best characteristics for investigating, *in vitro*, the effect(s) of weightlessness on mammalian cells (1, 15-17), and it has been extensively employed by a number of authors for modeling microgravity conditions on a variety of isolated cells (bone cells included) (12, 18, and references therein). The RCCS™ apparatus was, for those reasons, chosen for our study.

Objective of our work was to try to increase the reliability of the RCCS™ bioreactor-based culture methods, by establishing a protocol that, instead than on single, isolated bone-derived cells, should have permitted to investigate the long-term effects of modeled

microgravity on the complex, multicellular, intact bone tissue, that, by preserving the whole original microenvironment and microarchitecture, mirrors more closely the *in vivo* situation. It has been, in effect, extensively shown that tissue-specific cell phenotypes, functions and responses are strictly dependent on biochemical signals and cues deriving from their own microenvironment (16).

In a previous work (19), we suggested that the exposure of proximal rat tibial bone explants to modeled microgravity conditions generated by the RCCS™ bioreactor is consistent with the skeletal changes observed *in vivo* after spaceflight, and that the RCCS™ bioreactor-based culture can provide a reliable 3D *in vitro* method for whole bone tissue (organ) culture from adult organisms, suitable for research purposes. A quantitative assessment of the changes in mechanical properties of the bone explants was possible thanks to a numerical model based on the Cell Method (20), which was used for a comparative evaluation of the trabecular bone structures reconstructed by micro-CT. The explants, 1 to 3 for each experimental point, were harvested after 3 days and after 1, 2, 3 and 4 weeks of culture in the RCCS™ bioreactor. At that time, no data points at T0 - neither control points at T3-T4 - were available for the study, nor it was possible to perform statistical analyses of the results.

In a subsequent work, we compared the elastic properties of the trabecular structures of the fragments of rat tibial bone explants examined in (9) with those of samples kept for the same periods of time in a traditional, static, tissue culture system (i.e. subjected to Earth's gravity force, but not to the physiological loading, Figure 2) (21, 22). The mechanical properties of the structure of the samples (apparent elastic moduli, E) and the values of the trabecular bone volume fractions (BV/TV) obtained from this experimental procedure are illustrated in Figure 1. While well reflecting the expected difference between the elastic properties along the *xy* plane and those in the orthogonal *z-axis* (this last physiologically

subjected to the higher, gravity-dependent mechanical loading), the bone properties of the cultured samples did not exhibit a definite trend that could be related to the particular experimental condition, and, after 4 weeks of culture, the structural and mechanical properties of the samples were similar to those of the T0 controls, as reflected also by the morphological aspects of the samples (21).

In the present work, we evaluate the long-term effects of RCCS™-modeled *in vitro* weightlessness (3 to 4 weeks) on the morphology and on the structural elastic properties of tibial cancellous bone explants (proximal epiphyses) from young rats, and compare the results obtained with those from living animals of the same age, kept, in parallel, in physiological 1g-exposed conditions and loading. Eight samples were collected as controls (living animals under physiological 1g-exposed condition), and six samples, from different animals, were available for the culture in the RCCS™-modeled microgravity environment, allowing for a quantitative analysis of the results.

2 Materials and Methods

2.1 *Modeled microgravity conditions*

Modeled microgravity conditions were attained by the use of the Rotary Cell Culture System (RCCS™) bioreactor (Synthecon Inc., Houston, TX, U.S.A.), in 55 ml Slow Turning Lateral Vessel (STLV) culture chambers. Originally developed by N.A.S.A.'s technological research at JSC in U.S.A., RCCS™ is a slow-rotating clinostat, capable to generate, inside the culture chamber, a particular condition where the gravitational field is time-averaged to near zero over each revolution, thus negating the influence of gravitational sedimentation, and reproducing some specific aspects of microgravity (modeled microgravity) (15, 23). Figure 2 illustrates the working principle of RCCS™ bioreactor. Without going into the details, the typical hydrodynamic conditions created inside the culture vessel have been demonstrated,

also by our group, to be fundamental to permit the 3D long-term culture of cells/tissues of various origin (14, 24-28).

2.2 *Samples and culture conditions.*

In the present study, the structures of 14 rat tibial epiphyseal bone explants were analyzed (8 controls and 6 microgravity-exposed samples). As in our previous study (see 19), donor animals were young (7-8 weeks-old male *Sprague-Dawley*) rats (Harlan-Europe, Milan, Italy), from 200 to 225 g of body weight. Until being put to death (by decapitation, a few days after their arrival in the animal facility), the experimental animals were housed in a climate-controlled room (at about 22°C and 5% humidity), with a 12 h light-dark cycle, and they received tap water and standard laboratory rat diet *ad libitum*. All of the experimental procedure was authorized by the Italian Ministry of Health and by the University of Brescia's Animal Care Committee, in compliance with the Italian guidelines for experimental animal care (DL 04.03.2014 n.26) and with the EU Directive 2010/63/EU; local Prot. 02/08.

Eight control samples (CONTROL) were obtained from healthy, living animals: five were from young animals (7-8 weeks of age), and were collected at the beginning of the experimental procedure, and three, obtained at the end of the procedure, were from rats 4 weeks older. We considered all these samples as a homogeneous group of reliable controls, since no significant difference was observed by comparing their individual structural and mechanical properties (not shown).

Microgravity-exposed samples (RCCS) were six in number, and were obtained after 3 to 4 weeks of dynamic culture in the RCCS™ bioreactor (modeled microgravity). Again, no significant difference was observed by comparing their individual structural and mechanical properties (not shown). This result is in agreement with our previous findings (19).

Epiphyseal bone preparation, culture conditions, and sampling were performed as previously described (19). Briefly, with reference to bone culture conditions in the RCCS™ bioreactor, the bone samples were kept in hg D-MEM culture medium, supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic mix (1000 U/L penicillin G, 1000 µg/L streptomycin sulfate, 150 µg/L amphotericin B); the medium was refreshed twice a week. After 3 to 4 weeks of culture, the bone samples were harvested for further processing and analyses. The experimental plan is depicted in Figure 3.

For micro-CT analyses, all the bone samples were washed with phosphate buffered saline solution, fixed for 7 days with 10% buffered formaldehyde solution, left to air-dry, and kept at room temperature. Histo-morphological study (Hematoxilin & Eosin staining) was performed on the same samples that underwent micro-CT.

Chemicals: culture-related products were purchased from Life technologies (San Giuliano Milanese, Italy); all the other chemicals were of analytical grade purity, and were purchased from Sigma-Aldrich (Milan, Italy), unless otherwise indicated.

2.3 *Structural assessment*

2.3.1 Micro-CT and image processing

After being harvested, the bone structures were reconstructed by micro-CT at the SYRMEP beamline at Elettra (Elettra proposal 2008164), the synchrotron radiation facility in Trieste (Italy), at a 9-micron resolution. Synchrotron light imaging techniques, and particularly X-ray micro-tomography (micro-CT), combine the advantages of non-destructive techniques with a high spatial resolution. Micro-tomography consists in the acquisition of a large number of radiographic projections, captured at different angular positions of the sample (which is placed on a rotary table) with respect to the source of X-rays, usually completing a

180° rotation. By means of specific algorithms (in this case implementing the back-projection method), the different transverse sections (slices) are reconstructed from the angular projections, reproducing the sample structure, Figure 4(a). Compared to a conventional source, synchrotron light allows for improving image quality by avoiding beam-hardening artifacts. Moreover, the high coherence typical of synchrotron X-rays allows the application of phase contrast techniques, which enhance the visibility of the edges inside the sample and contribute to highlight the internal structure of the bone trabeculae (29).

Since the structural analyses are typically performed on cubic volumes of interest of 200-pixel side (1.8 mm), a special procedure was defined in order to identify the anatomically and structurally homologous portions in samples extracted from different individuals.

1. Each data set was rotated so that all reconstructed tibias were oriented in the same way, Figure 4(a):
    - z is the rotation axis of the micro-CT;
    - y is the axis connecting the tibia section vertex, which has a characteristic "heart" shape, with the center of the minimum circumscribed circle (MCC) encompassing the tibia section in the first slice of the stack;
2. Homologue z positions were identified, despite the lack of a clear anatomical common structure that can be used as a reference, so that the first slice was the first external to the cartilage growth line, where the trabecular bone occupies the entire tibia section and no other bone structures are detectable, Figure 4(b).

By superimposing a sequence of slices it is then possible to obtain a volumetric representation of the examined trabecular samples, Figure 4(c), which can be considered as a "virtual biopsy" of the experimental sample.

2.3.2 Morphological analyses

The trabecular bone principal anisotropy direction in our samples is *z*, Figure 4(d), and the Quant3D software (30) was used to confirm that all the volumes of interest were aligned along this direction. The threshold for the binary segmentation of the trabecular bone fraction was computed with the iterative algorithm implemented in the software and described in (31). The Mean Intercept Length (MIL) is a well-known method used to assess the anisotropy properties of a binary volume (32). The mean distance between two intersections of a linear grid with the bone-marrow interfaces (MIL) was computed for 2049 orientations and 1000 random points in each volume, and the principal directions of the MIL fabric tensors were confirmed to be aligned with the *z*-axis in each volume (Table 1).

TheQuant3D software was also used to compute the percentage trabecular bone volume fraction BV/TV, the trabecular number Tb.N and the trabecular thickness Tb.Th histograms in the same volumes of interest used for the structural analysis.

2.3.2 Cell Method model

The development of methods for the numerical modeling and the prediction of behavior is of a great interest for a wide class of materials with complex structures, which ranges from materials of industrial interest, such as short fiber reinforced composites or sintered alloys, to biological tissues (i.e. bone tissue) and novel bioengineered tissue structures. Cell Method numerical models have been developed for several applications, and often allow a significant reduction in computation requirements compared to conventional FEM analyses. The main characteristics of the method, originally proposed by Tonti (33), are:

- it allows a direct discrete formulation of physical laws, since no differential formulation is used to write the fundamental equations;
- Cell Method results are comparable with Finite Element Method ones;

- the method is applicable when discontinuities are present;
- the same characteristic length can be used for heterogeneities and mesh.

The details of the Cell Method formulation for elastostatics are discussed in detail in (33-35). A wide range of applications and works by several Authors is described in the publications listed at the website: http://discretephysics.dic.units.it.

Micro-mechanical numerical models based on the Cell Method have been developed for the quantitative assessment of the apparent elastic properties of porous materials (36-38), sintered alloys (39) and short fibre reinforced polyamide composites (40, 41). Models have also been developed for the analysis of the trabecular bone microstructures from planar radiographic images (42-44) and micro-CT reconstructions. In particular, the model, developed for the analysis of the trabecular bone microstructure, and described in detail by Cosmi and co-workers (19, 20), has been applied for the simulation of compression tests on the cubic volumes of interest in this work.

The principal steps can be summarized as follows:

1. each volume of interest is discretized using the same mesh (812,905 tetrahedral cells, 141,982 nodes);
2. an elastic, homogeneous and isotropic law was assumed in each cell;
3. the elastic modulus of each cell was determined by a scaling procedure based on the grey level in the tetrahedra vertexes and barycenter, having assumed for the trabecular structure an elastic modulus E=1 GPa, ν=0.3.

The apparent elastic modulus of the bone structures in the volumes of interest was then computed along the three coordinate axes. Computation time on an ordinary PC was about 2.5 hours for the complete analysis of a sample, including model creation, meshing, and solution in the three axes.

3 Results

Although the number of specimens available for each experimental point is limited, it is, nevertheless, sufficient to obtain comparative statistical values.

The results of the morphological analyses are summarized in Figure 5, where the values of the percentage trabecular bone volume fraction (*BV/TV*), the trabecular number (Tb.N), and the trabecular thickness - computed as the mean minimum measured length in all directions through a random point placed within bone (30) - (Tb.Th), studied in the micro-CT volumes of interest, are shown.

The percentage trabecular bone volume fraction BV/TV of the explants kept in the RCCS™ bioreactor (RCCS) (average 0.13±0.01, n= 6) presents a significant decrease (statistical t-test with $p < 0.01$) with respect to the controls (CONTROL) (average 0.17±0.02, n= 8). The average BV/TV loss is 22%.

The trabecular number (Tb.N) parameter confirmed the tendency observed for the BV/TV values. The CONTROL samples present a mean trabecular number Tb.N (average 7.4±1.2 mm-1, n= 8) significantly higher (statistical t-test with $p < 0.01$) than the RCCS™ ones (average 4.3±0.7 mm-1, n= 6). The average loss in Tb.N is more pronounced, 42%, than the trabecular bone volume loss. The correlation coefficient between the trabecular bone fraction and the trabecular number was high, $R^2 = 0.8838$.

However, no significant differences were observed in the mean trabecular thickness (Tb.Th) of the control explants (average 25.3±1.3 µm, n= 8) and of those kept in the RCCS™ bioreactor (average 28.0±3.3 µm, n= 6). These results are in complete agreement with those reported by Tavella et al. (10), and obtained, *in vivo*, by the analysis of weight-bearing bones of mice after a long-term exposure to near-zero gravity during spaceflight (9). Our findings, therefore, confirm that the RCCS™ culture method used to model microgravity conditions can

represent a reliable model for investigating, on ground, spaceflight effects on whole bone tissue.

A general degradation in the mechanical properties of bone samples is observable after 3-4 weeks of culture in the RCCS™ bioreactor under modeled microgravity conditions.

The apparent elastic modulus results indicate a higher stiffness along the *z-axis,* as anticipated, and lower elastic properties in the orthogonal *xy* plane, where the expected transversely isotropic behavior of the bone trabecular structure can be appreciated (Figure 6).

If we compare the mechanical properties of the RCCS™ bone explants with those of the normal cancellous bones of the controls, the apparent elastic moduli along the *z-axis* of the explants kept in the RCCS™ bioreactor (average 46.3±6.3 MPa, n= 6) presents a significant decrease (statistical t-test with $p < 0.05$) with respect to the controls (average 78.1±28.7 MPa, n= 8), with an average percentage loss of 41 % .The change in the *xy-plane* between CONTROL (average 27.7±11.6 MPa, n= 8) and RCCS™ bone explants (average 13.2±2.2 MPa, n= 6) is also significant (statistical t-test with $p < 0.05$), and amounts to 52%. It can be noted that the alterations in the apparent elastic moduli Ez, Ex, Ey are markedly more pronounced than those found in BV/TV and that the differences in the average transverse moduli are more marked than those in the principal modulus.

The modifications of BV/TV between the pooled CONTROL and the pooled RCCS™ samples were related to the respective differences in apparent elastic moduli with R^2=0.4498 (linear fit) for the in-vivo gravitational load direction z. A similar value was found the transverse *xy* direction, with R^2= 0.4441 (linear fit).

The histo-morphological analyses performed on the same samples, demonstrate microgravity-induced alterations, and are suggestive of an active bone remodeling; moreover, as illustrated in Figure 7, they show that in the RCCS™-based culture system the viability of

bone cells, as well as the gross native tissue architecture, were preserved up to the end of the experimental procedure.

4 Discussion and Conclusions

Bone preservation and repair are critically linked to the mechanical forces acting on the bone itself. Adaptive changes in bone tissue caused by changing loads have been already connected to structural and mechanical properties of bone tissue (45), while, more recently, a high correlation coefficient (80-90%) between structural and mechanical properties of samples of from normal and pathologic (osteoporotic, osteoarthrosic) cancellous human bone tissue was proved (46). Mechanical properties and internal structure are then important parameters that define bone quality and, based also on a progressively better understanding of the pathogenetic mechanisms of altered bone conditions, should enable diagnostics and adequate therapy of bone diseases.

Even if cell mechanics has been an active area of research for many years, the biological bases of cell response to mechanical stimuli are, in reality, largely unknown. New integrated approaches, as well as new experimental models, are, in consequence, needed in order to attain better understanding of bone physiopathology to this concern. This is particularly true for spaceflight-induced bone modifications, occurring in near-weightless conditions (microgravity) (47), where the biochemical data of astronauts and the histo-morphometric analysis of rat bones show that the change in bone mass could be a result of decreased bone formation in association with normal (or increased) bone resorption. Vico and co-workers already reported spaceflight data obtained on isolated bone cells (from rodents and humans), and demonstrated that cytoskeleton organization and mechanical stress should be involved in bone mass changes (48). More recently, Tavella and co-workers reported data obtained *in vivo*, on mice exposed for a long period to microgravity conditions on the

International Space Station, suggesting that space-related morphological alterations in weight-bearing bones may be due to both an increased bone resorption and a decreased bone deposition (10).

Other studies, conducted at the *macro*scale, considered the changes in the bone mineral density by means of well-established procedures, such as Dual Energy X-ray Absorptiometry or Quantitative Ultrasound. In (49), the substantial loss of both trabecular and cortical bone was demonstrated to be site-specific, also by measuring changes in the geometrical parameters describing distinct anatomical segments (i.e. femoral neck and vertebrae).

Observations conducted at the *micro*scale, carried out by detecting the activity and the interactions between bone cells, as deduced from histo-morphometric, biochemical and molecular analyses (see, for example, 50 and 51), demonstrated significant microgravity effects on bone cell shape, nuclear shape and architecture, and specific gene expression, proving that gravity *per se* may play a pivotal role in the regulation of osteoclasts/osteoblasts/bone marrow cells proliferation/function and differentiation processes.

In a novel approach, our work was aimed at investigating, at the *meso*-scale level, the structural effects of cancellous bone alterations due to long-term exposure of whole bone explants to simulated weightlessness. While the RCCS™ bioreactor has been efficiently used for cell and tissue culture for over twenty years now, its use for the dynamic, 3D culture of whole bone tissue from adult organisms had not been previously established, and represents an innovation, first described in 2009 by our group (rat bone explants) (19).

We have also recently demonstrated the relevance of our RCCS™ bioreactor-based tissue culture model in closely reproducing the *in vivo* situation in humans, in the case of long-term culture of Multiple Myeloma samples, derived from pathological human whole bone/bone marrow explants (28).

If compared to the previous results obtained by our group (19), the main original aspect of the present work consists of the fact that the former was a pure methodological study (which demonstrates the reliability of the experimental methods adopted and the feasibility of their integration), while this latter is a direct application of those methods. Unlike the first one, the current study gave rise to a statistically significant number of experimental results, includes morphometric analyses, and proves, at a time: i) the value of our *ex vivo* culture method that, strictly mirroring the *in vivo* situation (see 10), can be successfully applied for on ground studies of microgravity effects on whole bone tissue (which could be also of human origin; see (28)), and ii) the importance of our original integrated experimental approach that, coupling an innovative bone tissue culturing technique with a numerical method for computing the effect of environmental changes on adult bone metabolism, open novel perspectives for adding new knowledge to bone cells' physiology (response to loading) and for investigating, at deeper levels (e.g. biomechanical and biomolecular), the specific mechanism(s) of the decrease of bone quality during spaceflight, necessary prerequisite for the development of effective preventive/therapeutic countermeasures.

Furthermore, even if based on the use of whole bone explants, our *ex vivo* experimental method, which has been demonstrated by our group to be suitable also for normal/pathological samples of human origin (28), fully complies with the 3R's strategy, which - based on the principle of Russel & Burch (52) for the needs of "alternative" methods to animal use in biomedical research and testing - is aimed at improving ethical standards and animal welfare in *in vivo* experimental procedures.

The work here described also points out that an accurate representation of trabecular bone modifications cannot be achieved without considering the degradation of the mechanical characteristics of the trabecular bone structure, apart from the modifications in the trabecular

morphology and the decrease in the bone volume fraction. While, in this study, the trabecular bone fraction BV/TV and the trabecular number Tb.N appear to be somehow related, the changes in the apparent elastic moduli Ez, Ex, Ey are completely unrelated to the modifications found in BV/TV and in the trabecular number Tb.N. This result is quite interesting, since it confirms that the microstructural changes in the bone elastic properties cannot be ascribed to bone volume loss alone, but are indicative of structural modifications taking place, simultaneously, in the bone micro-architecture.

Even if, as with most *in vitro* systems, our experimental conditions neglect the influence of systemic factors (such as, for example, hormones), the tissue-culture strategy we propose, that preserves the original structural, cellular, and biochemical microenvironment of bone tissue (cell/matrix components and, likely, also the local network of growth factors' and cytokines' signaling), may really open new perspectives for adding new knowledge to bone cells physiology (and response to loading).

In conclusion, this interdisciplinary and integrated experimental approach, that, to our knowledge, has never been reported before, proved that our RCCS™ bioreactor-based culture method may be considered as the first reliable *ex vivo* model capable of reproducing, on ground, and, potentially, also in human tissue, the typical bone alterations that take place, *in vivo*, during long-term spaceflight.

Furthermore, this work points out that the degradation in bone structural performance (apparent mechanical properties, evaluated by numerical simulations able to quantify trabecular bone modifications) must be considered and that an accurate representation of bone quality requires an estimate of the mechanical response and cannot be limited to the morphological characterization.

Looking forwards, the exploitation of this innovative methodology within a human

context may also constitute a very promising research tool in order to properly investigate the long-term effect/s of environmental agents/conditions on bone physiology, to clarify the pathogenetic mechanisms of bone disorders, to test bone response to preventive and interventional therapeutic strategies for predicting clinical outcomes (to be applied, for example, to a wide range of primary/secondary osteoporotic diseases), and, finally, also to estimate the clinical relevance of novel bioengineered implantable tissue structures.

Acknowledgements

The authors wish to acknowledge a variety of contributions that, at different stages of this interdisciplinary research, were pivotal in different ways to this work. Franco Vittur, University of Trieste, provided the first samples used during the numerical model development and validation phases. Jennifer Boniotti applied her skilled technical help during the preparation of bone samples at Brescia University. Enzo Tonti, University of Trieste, shared precious advice on the Cell Method. Angiola Berenzi and Anna Benetti, Institute of Pathological Anatomy of the University of Brescia, provided the anatomo-pathological examination and diagnosis of bone sample histology. Diego Dreossi, Elettra synchrotron facility, contributed to image elaboration and model implementation. Nicola Sodini, Elettra synchrotron facility, assisted during the acquisition of images. Fabio Daneluzzi, Barbara Reggiani, Salvatore Scozzese, Patrizia Barone and several other students should also be acknowledged for their valuable contribution.

The Area Science Park SISTER project, founded by FVG Region, financially supported F.C. in the development of the numerical model used in this work. This work was also partly supported by EC funding from the EXERA project n. LSHTCT2006037168 to GM.

Conflict of interest statement

All the authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest, or non-financial interest in the subject matter discussed in this manuscript.

References

1. Pietsch J, Bauer J, Egli M, Infanger M, Wise P, Ulbrich C, Grimm D (2011) The effects of weightlessness on the human organism and mammalian cells. Curr Mol Med 11: 350-364.

2. LeBlanc AD, Spector ER, Evans HJ, Sibonga JD (2007) Skeletal responses to space flight and the bed rest analog: A review. J Musculoskelet Neuronal Interact 7: 33-47.

3. Turner RT (2000) Invited review: What do we know about the effects of spaceflight on bone? J Appl Physiol 89: 840-847.

4. Vico L, Collet P, Guignandon A, Lafage-Proust MH, Thomas T, Rehaillia M, Alexandre C (2000) Effects of long-term microgravity exposure on cancellous and cortical weight-bearing bones of cosmonauts. Lancet 355: 1607–1611.

5. Zhang P, Hamamura K, Yokota H (2008) A brief review of bone adaptation to Unloading. Genomics Proteomics Bioinformatics 6: 4-7.

6. Zérath E, Grynpas M, Holy X, Viso M, Patterson-Buckendahl P, Marie PJ (2002) Spaceflight affects bone formation in rhesus monkeys: A histological and cell culture study. J Appl Physiol 93: 1047-1056.

7. N.A.S.A. (National Aeronautics and Space Administration): Human Research - Human Research Roadmap, HRR: risks, http://humanresearchroadmap.nasa.gov/Risks/ (lastly accessed on December 26, 2014).

8. N.A.S.A. (National Aeronautics and Space Administration): Human Research Program Integrated Research Plan (HRP-47065) REV F: 1-43 (2014).

9. Cancedda R, Liu Y, Ruggiu A, Tavella S, Biticchi R, et al. (2012) The Mice Drawer System (MDS) Experiment and the Space Endurance Record-Breaking Mice, PLoS One, 7(5): e32243. DOI: 10.1371/journal.pone.0032243.

10. Tavella, S. Ruggiu A, Giuliani A, Brun F, Canciani B, et al. (2012) Bone turnover in wild type and pleiotrophin-transgenic mice housed for three months in the International Space Station (ISS). PLoS One. 2012;7(3):e33179 DOI: 10.1371/journal.pone.0033179.

11. Ziambaras K, Civitelli R, Papavasiliou SS (2005) Weightlessness and skeleton homeostasis, Hormones 4(1): 18-27.

12. Ulbrich C, Wehland M, Pietsch J, Aleshcheva G, Wise P, van Loon J, Magnusson N, Infanger M, Grosse J, Eilles C, Sundaresan A, Grimm D (2014) The impact of simulated and real microgravity on bone cells and mesenchymal stem cells, BioMed Research International, vol 2014, Article ID 928507, 15 pages, http://dx.doi.org/10.1155/2014/928507.

13. Bucaro MA, Zahm AM, Risbud MV, Ayyaswamy PS, Mukundakrishnan K, Steinbeck MJ, Shapiro IM, Adams CS (2007) The effect of simulated microgravity on osteoblasts is independent of the induction of apoptosis. J Cell Biochem 102: 483-495.

14. Rucci N, Rufo A, Alamanou M, Teti A (2007) Modeled micro-gravity stimulates osteoclastogenesis and bone resorption by increasing osteoblast *RANKL/OPG ratio.* J Cell Biochem 100: 464-473.

15. Ayyaswamy PS, Mukundakrishnan K (2007) Optimal conditions for simulating microgravity employing NASA designed rotating wall vessels. Acta Astronaut 60: 397–405.

16. Mazzoleni G, Di Lorenzo D, Steimberg N (2009) Modelling tissues in 3D: the next future of pharmaco-toxicology and food research? Genes & Nutr 4(1): 13-22.

17. N.A.S.A. (National Aeronautics and Space Administration) Educational brief: NASA's Bioreactor: Growing Cells in a Microgravity Environment, EB-2002-12-187-MSFC, available at http://er.jsc.nasa.gov/sEh/cell_growth_in_zero_g.pdf, (lastly accessed on December 26, 2014).

18. Synthecon, Inc., at http://www.synthecon.com/bibliography.html (lastly accessed on December 26, 2014).

19. Cosmi F, Steimberg N, Dreossi D, Mazzoleni G (2009) Structural analysis of rat bone explants kept in vitro in simulated microgravity conditions. J Mech Behav Biomed Mater 2 (2): 164-172.

20. Cosmi F, Dreossi D (2007) Numerical and experimental structural analysis of trabecular architectures. Meccanica 42: 85-93.

21. Cosmi F, Steimberg N, Dreossi D, Mazzoleni G (2012) Quantitative structural evaluation of bone tissue explants kept in different experimental conditions. In: Bedzinski, R, Petrty M editors. Lecture Notes of the ICB Seminars, 86, "Current trends in development of implantable tissue structures", pp. 34-39.

22. Steimberg N, Benetti A, Berenzi A, Mazzoleni G (2011) Simulated microgravity induces complex histomorphological alterations on the osteo-articular system: an in vitro study on bone tissue explants kept in culture by the use of the RCCS™ bioreactor, In Proceedings of the "V National Congress of the Italian Society of Space Biomedicine and Biotechnology": Spazio, la Nuova Frontiera per l'Umanità; ISSBB editor, Padova (I), 28.

23. Klaus DM (2001) Clinostats and bioreactors. Gravit Space Biol Bull 14: 55-64.

24. Darling EM, Athanasiou KA (2003) Articular Cartilage Bioreactors and Bioprocesses Tissue Eng 9(1): 9-26.

25. Steimberg N, Boniotti J, Mazzoleni G (2010) 3D culture of primary chondrocytes and bone/cartilage tissue explants in simulated microgravity. In Yarmush ML, Langer RS, editors. Methods in Bioengineering: Alternative Technologies to Animal Testing. Artech House, USA. pp. 205-225.

26. Mazzoleni G, Steimberg N (2010) 3D culture in microgravity: a realistic alternative to experimental animal use. ALTEX 27: 321-324.

27. Mazzoleni G, Steimberg N (2012) New models for the in vitro study of liver toxicity: 3D culture systems and the role of bioreactors. In: Dr. Tyshenko MG, editor. The continuum of Health Risk Assessment. Intech Open Access Publisher, Rijeka, Croatia, pp. 161-194.

28. Ferrarini M, Steimberg N, Ponzoni M, Belloni D, Berenzi A, Girlanda S, Caligaris-Capio F, Mazzoleni G, Ferrero E (2013) Ex-vivo dynamic 3-D culture of human tissues in the RCCS™ bioreactor allows the study of multiple myeloma biology and response to therapy. PLoS One 8(8): e71613. doi:10.1371/journal.pone.0071613

29. Ritman EL (2004), Micro-computed tomography-current status and developments, Annu Rev Biomed Eng 6:185–208.

30. Ketcham RA, Ryan TM (2004) Quantification and visualization of anisotropy in trabecular bone. J Microsc 213:158-171.

31. Ryan TM, Ketcham RA (2002) The three dimensional structure of trabecular bone in the femoral head of strepsirrhine primates. J Hum Evol 43: 1-26.

32. Odgaard A (1997) Three-dimensional methods for quantification of cancellous bone architecture. Bone, 4, 315-328.

33. Tonti E (2001) A Direct Discrete Formulation of Field Laws: The Cell Method. CMES-Comp Model Eng & Sci 2(2): 237-258.

34. Cosmi F (2001) Numerical Solution of Plane Elasticity Problems with the Cell Method. CMES-Comp Model Eng & Sci 2: 365-372.

35. Tonti E, Zarantonello F (2009) Algebraic Formulation of Elastostatics: the Cell Method. CMES-Comp. Model Eng & Sci 39: 201-236.

36. Cosmi F, Di Marino F (2001) Modelling of the Mechanical Behaviour of Porous Materials: A New Approach. Acta Bioeng Biomech 3: 55-65.

37. Cosmi F (2003) Numerical Modeling of Porous Materials Mechanical Behaviour with the Cell Method. Proc. of Second M.I.T. Conference on Computational Fluid and Solid Mechanics, Massachusetts Institute of Technology, Cambridge, MA, U.S.A., June, 17-20

38. Cosmi F (2004) Two-dimension estimate of effective properties of solid with random voids. Theoretical and Applied Fracture Mechanics. 42: 183-186.

39. Cosmi F (2011) A Cell Method model for sintered alloys. CMES-Comp Model Eng & Sci 74 (3&4): 269-282.

40. Cosmi F (2011) Local anisotropy and elastic properties in a short glass fibre reinforced polymer composite. Strain 47: 215–221.

41. Cosmi F (2011) A micro-mechanical model of the elastic properties of a short fibre reinforced polyamide. Procedia Engineering: 10: 2135-2140.

42. Cosmi F (2008) *Method to identify the mechanical properties of a material.* Patent: USA No. 10509512, deposited by University of Trieste.

43. Cosmi F, Dreossi D (2007) The Application of the Cell Method in a Clinical Assessment of Bone Fracture Risk. Acta Bioeng Biomech 9(2): 35-39.

44. Cosmi F, Mazzoleni G (2014) Evaluation of the structural quality of bone in a case of progressive osteoporosis complicating a CRPS syndrome of the upper limb. J Mech Behav Biomed Mater 29: 517-528.

45. Kabel J, Odgaard A, van Rietbergen B, Huiskes R (1999) Connectivity and the elastic properties of cancellous bone, Bone, 24(2): 115-120.

46. Nikodem A, (2012) Correlations between structural and mechanical properties of human trabecular femur bone, Acta of Bioengineering and Biomechanics, 14(2): 37-46.

47. Carmeliet G, Bouillon R (1999) The effect of microgravity on morphology and gene expression of osteoblasts in vitro. FASEB J 13: 129-134.

48. Vico L, Lafage-Proust MH, Alexandre C (1998) Effects of gravitational changes on the bone system in vitro and in vivo. Bone 22: 95-100.

49. Lang T, LeBlanc AD, Evans H, Lu Y, Genant H, Yu A (2004) Cortical and trabecular bone mineral loss from the spine and hip in long duration spaceflight. J Bone Miner Res 19: 1006-1012.

50. Hughes-Fulford M, Rodenacker K, Jütting U (2006) Reduction of anabolic signals and alteration of osteoblast nuclear morphology in microgravity. J Cell Biochem 99: 435-449.

51. Gershovich PM, Gershovich YG, Buravkova LB (2013) Molecular genetic features of human mesenchymal stem cells after their osteogenic differentiation under the conditions of microgravity. Human Physiology 39 (5): 540-544.

52. Russel WMS, Burch RL (1959) The Principles of Humane Experimental Technique, Methuen & Co. Ldt., London: pagg. 169-154, ISBN 0900767782, and now retrievable on http://altweb.jhsph.edu/pubs/books/humane_exp/het-toc.

Tables

Table 1. Properties of the eigenvectors of the major principal axis of the MIL fabric tensor

	CONTROL	**RCCS**					
		$\cos\alpha$	$\cos\beta$	$\cos\gamma$	$\cos\alpha$	$\cos\beta$	$\cos\gamma$
Average		-0.04	-0.09	0.99	0.04	-0.08	0.99
Standard deviation		0.10	0.10	0.01	0.04	0.09	0.01

Legends to figures:

Figure 1. **A**: Average values of apparent elastic moduli (*E*) of the trabecular structure of bone samples kept in static culture conditions for 1 to 4 weeks: *Ez* (along *z-axis*, principal direction, *in vivo* gravitational load direction), *Ex* and *Ey* (orthogonal transversal directions). **B**: Average values of trabecular bone volume fraction, BV/TV, of the same samples as in A. *E* and *BV/TV* were calculated in the micro-CT volumes of interest. *: control value (T0).

Figure 2. Experimental culture conditions and forces acting on bone explants. In the RCCS™ bioreactor samples are maintained in a three-dimensional (3D) and dynamic microenvironment (orbital revolution, ω); in these conditions the force acting on the samples, resulting from gravitational field (Fg), centrifugation (Fc), and fluid drag (Fd), is time-averaged to near zero over each revolution, thus negating the influence of gravitational sedimentation, and mimicking some aspects of microgravity (modeled microgravity). In traditional static culture, on the contrary, bone explants are kept in a static and "bi-dimensional" (2D) microenvironment (plastic surface), where they are mainly (and almost only) exposed to the Earth's gravitational force.

Figure 3. Experimental plan.

Figure 4. Identification of the volumes of interest: in (a) the *xy* plane, (b) the plane in the *z* direction, (c) 3D representation of the trabecular structure of one sample.

Figure 5. Percentage trabecular bone volume fraction *BV/TV*, trabecular number Tb.N (1/mm) and trabecular thickness Tb.Th (micron, computed as the mean minimum measured length in all directions through a random point placed within bone) in the micro-CT volumes of interest.

Figure 6. Average values of apparent elastic moduli of the trabecular structure (MPa) in the micro-CT volumes of interest: *Ez* (along *z-axis*, principal direction, *in vivo* gravitational load direction), *Ex* and *Ey* (transversal directions).

Figure 7. Representative histological features of experimental bone samples (after µCT analysis). Photo-micrographs of Control_T0 and RCCS_4wks bone samples, stained with Hematoxilin & Eosin solution (4X magnification). Yellow circles indicate analogous areas of the samples, and show how, *in vitro,* modeled microgravity condition induces typical alterations of tissue microstructure, while preserving cell viability. Bm = bone marrow; Tb = trabecular bone; Gp = growth plate.