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Original Paper

Effects of Simulated Microgravity on Muscle Stem Cells Activity

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Key Words
Muscle stem cells • Simulated Microgravity • BMP-2 • Myostatin • Degeneration phenomena

Abstract
Background/Aims: The study of the effects of simulated microgravity on primary cultures of human satellite cells represents a reliable model for identifying the biomolecular processes involved in mechanic load-related muscle mass loss. Therefore, this study aims to investigate the role of myostatin and Bone Morphogenetic Protein-2 in human satellite cells response to simulated microgravity condition. ***Methods:*** In order to identify the main molecules involved in the phenomena of degeneration/regeneration of muscle tissue related to the alteration of mechanic load, we performed a morphological and immunohistochemical study on 27 muscle biopsies taken from control, osteoporotic and osteoarthritic patients, underwent hip arthroplasty. For each patient, we set up primary satellite cell cultures subjected to normogravity and simulated microgravity (110h) regimens. Cellular functionality has been studied through a morphological evaluation performed by optical microscopy, and an ultrastructural evaluation carried out by transmission electron microscopy. Furthermore, we evaluated the expression of Bone Morphogenetic Protein-2 and myostatin through immunocytochemical reactions. ***Results:*** Our results showed that in the very early phases of simulated microgravity condition the satellite cells are more active than those subjected to the normogravity regime, as demonstrated by both the increase in the number of myotubes and the significant increase in the expression of Bone Morphogenetic Protein-2 in all experimental groups. However, with prolongated exposure to simulated microgravity regime (>72h), satellite cells and new formed myotubes underwent to cell death. It is important to note that, in early phases, simulated microgravity can stimulate the formation of new myotubes from satellite cells derived by osteo-

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porotic patients. Furthermore, we observed that simulated microgravity can induce changes in myostatin expression levels by group-dependent variations. ***Conclusion:*** The results obtained allowed us to hypothesize a possible molecular mechanism of response to simulated microgravity, confirming the importance of Bone Morphogenetic Protein-2 and myostatin in the physio-pathogenesis of muscle tissue. In addition, these data can lay the foundation for new therapeutic approached in the prevention/cure of osteoporosis and sarcopenia.

Introduction

On Earth, organisms are constantly subject to contact forces that provide a series of mechanical stimulations essential for the function of many physiological systems.

The predominant terrestrial conditions, that differ from those found in space, are a) the presence of a geomagnetic field that deflects charged particles, b) the presence of atmosphere and c) the force of gravity. In space, this lack of normal gravity with the consequent loss of mechanical stimulation are responsible for the various changes that alter the normal physiology of cells and tissues [1, 2]. These changes affect morphology and cell growth through both an alteration of signaling pathways and a modification of gene expression [3].

It is known that the absence of loading forces is responsible for many of the physiological issues that astronauts manifest following spaceflights. In particular, the absence of loading forces induces a quickly tissues degeneration precluding long-term mission. The main damages induced by the gravitational environment include loss of bone [4-6] and muscle mass [7-8], cardiovascular dysfunction [9-10], possible alteration in the fracture healing processes [11-12] and wounds repair [13], impaired immune response [14-15].

Many of changes observed in astronauts during spaceflights recall the onset and progression of diseases associated with aging on Earth, although they occur much faster. Therefore, it is possible that changes observed in response to simulated microgravity may share the common physiological basis with some of physiological adaptations typical of aging, such as the loss of bone and muscle mass that characterizes the main musculoskeletal disorders, such as osteoporosis, arthrosis and sarcopenia.

In this regard, several studies have been conducted to evaluate effects of simulated microgravity on the musculoskeletal system. It is known that in bone tissue, simulated microgravity condition causes a loss of mass associated with an alteration of the balance between osteoblast and osteoclast activities, being able to stimulate the reabsorbing activity of osteoclasts and to inhibit the formation of new bone by osteoblasts [4]. This imbalance causes a loss of bone mass in astronauts at a rate of 0.5% to 1.5% per month [16].

In microgravity conditions, a consistent and associated reduction in muscle mass can also be observed. The absence of load forces during spaceflight is known to cause muscle atrophy, changes in muscle fiber composition and gene expression, as well as a reduction in regenerative muscle growth [8]. However, these processes have mainly been studied in rodents for short periods of time, while little is known about long-term effects of microgravity in human muscle, especially for what concern the role and activity of satellite muscle stem cells.

Satellite cells play an indispensable role in muscle regeneration. The self-renewing proliferation of these cells not only contributes to the maintenance of the stem cell population, but also provides numerous myogenic cells, which proliferate, differentiate, merge and induce the formation of new myofibers, thus promoting the reconstitution of a contractile apparatus functional [17]. The loss of satellite cells and/or their degeneration could be related to the altered muscle metabolism that characterizes osteoporotic patients.

In a previous study [18], we demonstrated that the activity of satellite cells was influenced by Bone Morphogenetic Proteins (BMPs) expression, which play a key role in controlling mass and muscle regeneration [19-21]. In particular, we recent demonstrated an association between BMP-2 and BMP-4 expression and the activity of satellite cells [18]. We also hypothesized that the quality of muscle tissue depends on the balance between BMPs

signaling and myostatin signaling [22]. Myostatin is known to affect muscle mass through negative regulation of myogenesis [23]. Indeed, *in vitro* experiments have shown that this myokine can block the proliferation of myoblasts, as well as the proliferation and renewal of satellite cells [24].

Starting from these considerations, this study aims to investigate the role of myostatin and BMP-2 in human satellite cells response to simulated microgravity condition.

Materials and Methods

Patients

In this study, we enrolled 9 patients underwent hip arthroplasty for medial femoral fracture (OP) and 9 patients underwent hip arthroplasty for osteoarthritis (OA) in the Orthopedic Department of "Tor Vergata" University. Moreover, we enrolled 9 patients underwent hip arthroplasty for high-energy hip fracture of the femoral head as a control group (CTRL).

Exclusion criteria were history of cancer, myopathies or other neuromuscular diseases or chronic administration of corticosteroid for autoimmune diseases (more than 1 month), diabetes, alcohol abuse, viral chronic infections (HBV, HCV, HIV), previously orthopedic surgical implants.

Patients were divided into OP and OA according to DEXA, *T*-score and radiographic assessment by Kellgren-Lawrence scale [25].

Bone Mineral Density Evaluation

Bone mineral density evaluation (DXA) was performed with a Lunar DXA apparatus (GE Healthcare, Madison, WI, USA). Lumbar spine (L1-L4) and femoral (neck and total) scans were performed, and bone mineral density (BMD) was measured according to manufacture recommendations [26]. Dual-energy X-ray absorptiometry measures BMD (in grams per square centimeter), with a coefficient of variation of 0.7%. For patients with cervical femoral fragility fracture, BMD was measured on the limb opposite to the fracture side. Instead, for the other patients, all measurements were performed on the nondominant side, while participants lay supine on an examination table with their limbs abducted away from the trunk [27]. DXA exam was performed 1 day before surgery for OA patients, and 1 month after surgery for OP and CTRL patients. The results were expressed as T-scores.

Radiological Analysis

Hip radiographs of all patients were obtained using a standard protocol [28]. Two orthopedists independently assessed all radiographs using the Kellgren-Lawrence (K-L) radiographic atlas [29]. Disagreements between the two orthopedists were resolved by consulting a third orthopedist. This system is used to classify the severity of osteoarthritis (OA) using five grades. Patients with a grade of K-L ≥ 2 were considered osteoarthritic.

Isolation and culture of primary human satellite cells

Primary cultures of satellite cells were isolated from the upper portion of the vastus lateralis muscle taken from patients underwent hip arthroplasty. Cell cultures were established in monolayer according to protocol of Valerie Askanas [30]. Muscle tissues were cleaned and transfer in culture dishes with Conditioning Media (CM) (Medium 199 containing Earle's Salts, stable Glutamine, 25 mM HEPES, 42% FBS and 1.5% amphotericin B (Biowest, Nuaillé-France)), and incubated at 37°C overnight. After adding 25% Human Plasma (HP) to the CM, culture dishes were incubated again for 7 days at 37°C checking for fibroblast outgrowth. Muscle tissues were removed from clotting media and cut up in 1 mm pieces, which were placed in gelatin-plasma coated dishes (1.5% gelatin). After adding F14 medium (DMEM F14 supplemented with 15% FBS and 0.08% amphotericin B (Biowest, Nuaillé-France), Penicillin-Streptomycin (Sigma Chemical Co., St. Louis, MO, USA), stable Glutamine (Biowest, Nuaillé-France), 0.01 mg/ml Human Insulin, 0.05 µg/ml FGF and 0.01 µg/ml EGF), culture dishes were incubated at 37°C for 5-7 days. After substantial growth, tissues were removed, and satellite cells were placed on new culture dishes coated with 0.2% gelatin. As soon as cells started to fuse into myotubes, they were washed with Hank's solution without calcium

and magnesium, and then switched in medium containing F14, 5% FBS, PSF and Insulin but no growth factors. Cell cultures were fed twice a week until use for experimental procedure.

Simulation of microgravity by Random Positioning Machine (RPM)

The influence of the force of gravity on eukaryotic cells was assessed using the desktop RPM system (Airbus Defence and Space Netherlands B.V.) [31]. All experiments were carefully planned according to procedures previously described [32]. The rotating frame of the desktop RPM was placed inside an ordinary cell culture CO_2 incubator. The software responsible for controlling the motion of RPM employed a tailored algorithm, which rotated with a random speed in such a way that the mean gravity vector reliably converged to zero over time, and it concurrently reduced fluid motion in the culture flask (Fig. 1). The samples were positioned compactly in the center of rotation, in order to avoid artifacts and to minimize centrifugal acceleration. All cell samples were carefully processed for *in vitro* cultivation. We used 24-well plates sealed with dialysis membrane (Visking Medicell International Ltd, Liverpool Road - London code DTV12000.06.000 MWCO 12/14 Kdalton). The dialysis membrane was deposited on the convex liquid meniscus of the medium inside the well, allowing it to be sealed and thus preventing the formation of air bubbles. The nitrocellulose discs were fixed to the support by means of a rubber ring. Plates were placed beside the RPM machine so that all samples shared identical culture conditions. Since most of the ground-based simulated microgravity research platforms are not vibration free, high-performance microscopy has not been applicable in Live Cell Imaging under RPM. Therefore, studies involving cell imaging have been carried out after chemical fixation of the cell. This approach implies a series of static shots, which reproduce dynamic events occurring in cells in response to microgravity exposure.

Morphological analysis

Satellite cells placed on culture dishes coated with 0.2% gelatin were fixed with 4% paraformaldehyde for 4h. Culture dishes were stained with toluidine blue and the morphological evaluation was blindly performed by two pathologists.

Immunohistochemistry

Immunohistochemical characterization was performed on culture dishes to assess BMP-2 and myostatin expression in all primary cultures of satellite cells. Briefly, cell samples were pretreated with EDTA citrate, pH 7.8 for 30 min at 95°C, and then incubated for 1h with rabbit monoclonal anti-BMP-2 (clone N/A; Novus Biologicals, Littleton, CO, USA) or with rabbit monoclonal anti-myostatin (clone ab134682, AbCam, Cambridge, UK).

Washings were performed with PBS/Tween20 pH 7.6 (UCS Diagnostic, Rome, Italy); reactions were revealed by horseradish peroxidase (HRP)-3, 3' diaminobenzidine (DAB) Detection Kit (UCS Diagnostic, Rome, Italy). To assess the background of immunostaining, we included a negative control for each reaction by incubating the sections with secondary antibodies (HRP) and a detection system (DAB). The immunohistochemical reaction was semi-quantitatively evaluated by assigning a score from 1 to 3 according to the number of positive myotubes. Results were showed as percentage of positive myotubes.

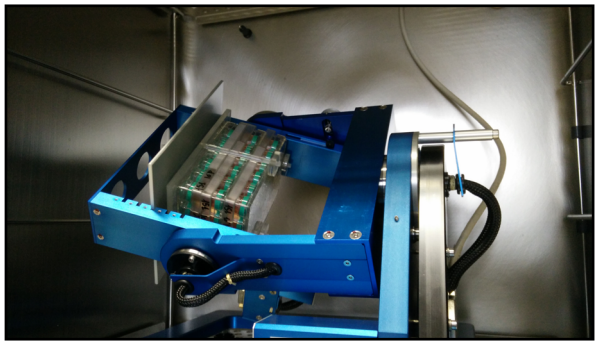

Fig. 1. Random Positioning Machine (RPM). The rotating frame of the desktop RPM was placed inside a CO_2 incubator for cell culture, and the movements of RPM were controlled by software through a specific algorithm. The culturing media were sealed with a transparent membrane, which was pressed to completely remove air bubbles from the culture chamber. Plates were placed beside the RPM machine so that all samples shared identical culture conditions.

Transmission Electron Microscopy (TEM)

Cell samples on culture dishes were fixed in 4% paraformaldehyde and post-fixed in 2% osmium tetroxide [33]. Culture dishes were washed with phosphate buffer 0.1 M, and dehydrated by a series of incubations

in 30%, 50%, 70%, ethanol. Dehydration was continued by incubations in 95% ethanol, absolute ethanol and propylene oxide. Culture dishes were incubated with 1:1 Epon-propylene oxide solution for 30 min, 3:1 Epon-propylene oxide solution for 30 min, and Epon absolute for 3h. Embedding beam capsules were placed over the culture dishes and incubated for 24h at 60°C. Finally, beam capsules were detached from the culture dishes with thermal shock (liquid nitrogen). All samples were studied by TEM Hitachi H-7100.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 Software (La Jolla, CA, USA). Clinical data were analyzed by Mann-Whitney test. Immunohistochemistry data were analyzed by one-way ANOVA and Mann-Whitney test and were expressed as mean value with standard deviation (n ± SD). The difference between groups was considered statistically significant at $p<0.05$.

Results

Clinical Evaluation

Clinical and instrumental evaluation allowed us to characterize patients enrolled in this study (Table 1). The OP group included 9 patients with fragility hip fracture, *T*-score ≤ -2.5 SD and K-L score from 0 to 1. The OA group included 9 patients with radiographic evidence of hip OA, with a K-L score 3 or 4 and *T*-score ≥ -2.5 SD. There was no discrepancy for age, sex and comorbidities in the two groups (mean age in years: OA 73.88 ± 3.48 vs OP 76.65 ± 1.50). CTRL patients were characterized by a *T*-score ≥ −1.0 SD and K-L score from 0 to 1.

Table 1. Main characteristics of CTRL, OA and OP patients

Parameters	CTRL	OA	OP	T-Test (Mann-Whitney Test)
Age (years)	46.11 ± 2.65	73.88 ± 3.48	76.65 ± 1.50	OP vs OA NS (p=0.11); OP vs CTRL, p<0.001; OA vs CTRL, p<0.001
BMI (Kg/m²)	22.05 ± 2.60	27.05 ± 0.60	22.36 ± 1.34	p=0.0005
T-score (L1-L4)	0.85 ± 0.01	-0.71 ± 0.40	-2.9 ± 0.15	p=0.0005
T-score (neck)	0.15 ± 0.08	-0.1 ± 0.15	-2.8 ± 0.16	p=0.0005

Morphological Analysis

Morphological analysis performed by optical microscopy allowed us to evaluate the cellular composition of primary cultures of human satellite cells (Fig. 2). All cultures were free from contamination by pathogens (bacteria, fungi, algae, etc.) and/or cells of different histo-type (such as fibroblasts).

In normogravity conditions, satellite cell cultures of the different experimental groups showed peculiar morphological characteristics. In particular, samples derived from CTRL patients (Fig. 2a) were characterized by the presence of numerous myotubes and rare single satellite cells. Similarly, to what observed in CTRL group (Fig. 2b), we noted a heterogeneous population formed by both myotubes

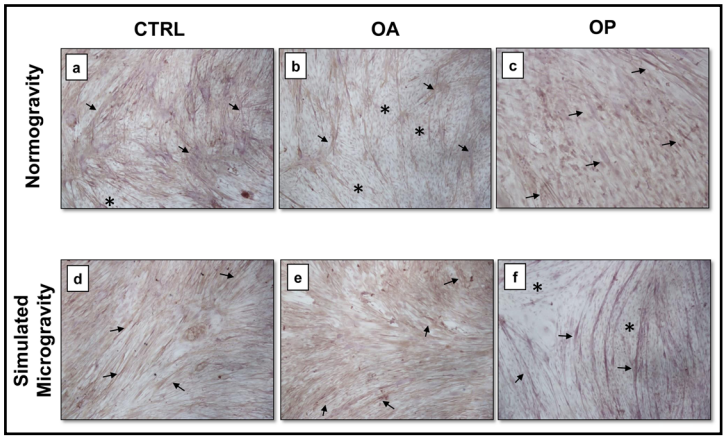

Fig. 2. Morphological analysis of primary cultures of human satellite cells in normo- and simulated microgravity conditions. In normogravity conditions, samples derived from CTRL (a) and OA (b) patients were characterized by the presence of numerous myotubes (arrows) and rare satellite cells (asterisks); in OP patients (c), primary cultures were mainly formed by single satellite cells (asterisks) and rare myotubes (arrows). In simulated microgravity conditions, a significant increase in myotubes (arrows) in all experimental groups (d,e,f) was observed.

and single satellite cells in cell cultures derived from OA patients. On the contrary, primary cultures obtained from OP patients displayed (Fig. 2c) single satellite cells and rare myotubes.

Noteworthy, satellite cells cultures subjected to a simulated microgravity regime (110h) showed the several morphological modifications. Specifically, in all experimental groups (Fig. 2d-f) we observed a significant increase in myotubes (CTRL 60% to 90%; OA 50% to 90%; OP 5% to 50%). In addition, morphological analysis showed numerous cell debris and areas of necrosis in all investigated satellite cells cultures subjected to a simulated microgravity regime. At least, signs of cell degeneration, such as the cytoplasmic vacuolization of myotubes, were observed.

Immunohistochemistry

The presence and activity of satellite cells were studied by using both BMP-2 and myostatin expression. Their expression was evaluated by counting the number of positive myotubes on 25 high-power field (HPF).

Evaluation of BMP-2 expression (Fig. 3). In normogravity conditions, we observed a significant increase in BMP-2 expression in primary cultures of CTRL patients (102.2 ± 6.5) compared to those of OA (94.2 ± 3.4) and OP (11.8 ± 2.4) patients. On the contrary, in simulated microgravity conditions, we observed a significant increase in BMP-2 expression in all experimental groups (CTRL 212.8 ± 17.9 vs OA 191.9 ± 3.8 vs OP 93.6 ± 4.8). In both experimental conditions, significant difference was observed between CTRL and OP groups ($p<0.0001$) and OA and OP patients ($p<0.0001$).

Evaluation of myostatin expression (Fig. 4). In cell cultures exposed to the normogravity regimen, we observed an age-dependent myostatin expression. In fact, an increase in myostatin expression was observed in both OP and OA patients respect to CTRL (OP 292.4 ± 29.4 vs OA 88.6 ± 12.1 vs CTRL 16.3 ± 3.5, $p<0.0001$). Noteworthy, primary cultures derived from muscle tissue of OP patients showed the highest levels of myostatin expression. We also observed that simulated microgravity was able to induce changes in myostatin expression levels by group-dependent variations. In particular, we observed a significant reduction in myostatin expression in OP patients (116.2 ± 14.4) if compared with both CTRL (145.2 ± 13.5) and OA (176.1 ± 16.8) groups (OA vs OP, $p<0.05$).

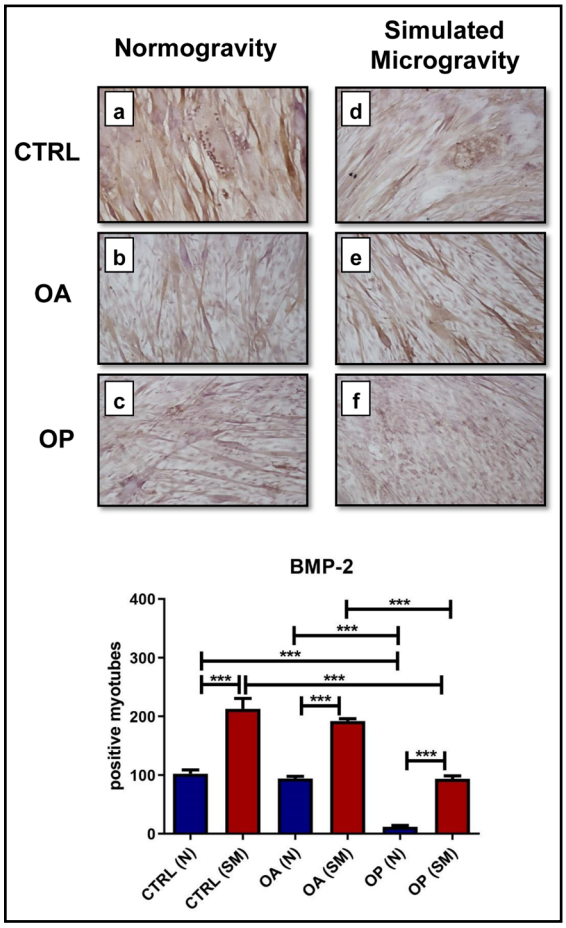

Fig. 3. Evaluation of BMP-2 expression. In normogravity conditions, a significant increase in BMP-2 expression in primary cultures of CTRL patients (102.2 ± 6.5) compared to those of OA (94.2 ± 3.4) and OP (11.8 ± 2.4) patients was observed (CTRL vs OP and OA vs OP, $p<0.0001$). In simulated microgravity conditions, a significant increase in BMP-2 expression in all experimental groups (CTRL 212.8 ± 17.9 vs OA 191.9 ± 3.8 vs OP 93.6 ± 4.8) was observed (CTRL vs OP and OA vs OP, $p<0.0001$). Note that a significant statistical difference was reported between the two CTRL groups ($p<0.0001$), the two OA groups ($p<0.0001$), and the two OP groups ($p<0.0001$). For each patient, the experiment was conducted in triplicate (n=27/group).

Transmission Electron Microscopy (TEM)

TEM analysis was performed to identify satellite cells according to their ultrastructural characteristics (Fig. 5). Cultures grown in normogravity showed viable and well-differentiated satellite cells, with no signs of cell death (Fig. 5a-f). However, TEM analysis surprisingly showed that myotubes previously described in cultures subjected to simulated microgravity regime were characterized by evident signs of cell degeneration (Fig. 5g-i). In particular, we observed satellite cells and myotubes rich in lipofuscin granules and electrondense cytoplasm in all cell cultures. Furthermore, in the cytoplasm of these cells, it was not possible to distinguish various cellular organelles. At least, dystrophic calcifications, a sign of cell death, were also observed.

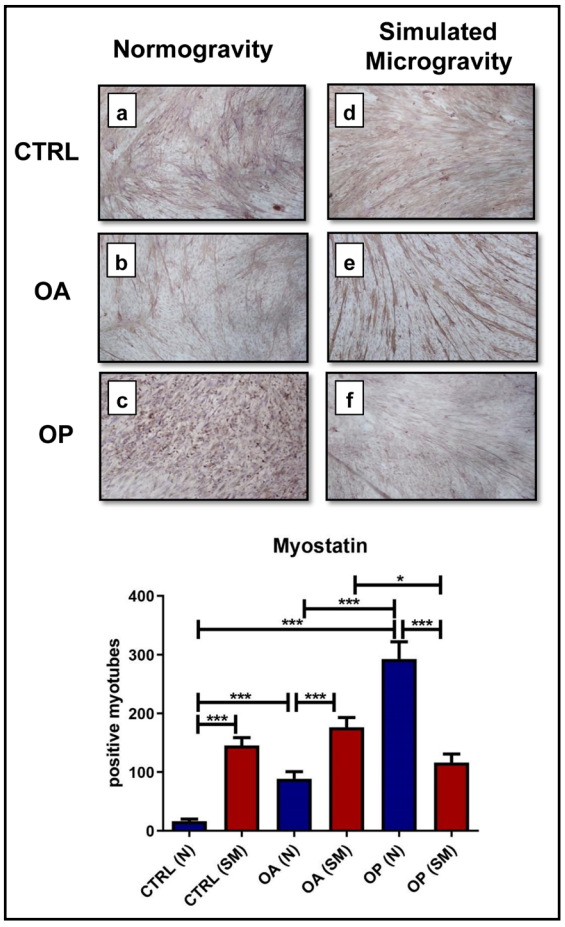

Fig. 4. Evaluation of myostatin expression. In normogravity conditions, a significant increase in myostatin expression in primary cultures of OP and OA patients respect to CTRL patients (OP 292.4 ± 29.4 vs OA 88.6 ± 12.1 vs CTRL 16.3 ± 3.5) was observed (OP vs OA, OP vs CTRL and OA vs CTRL, $p<0.0001$). In simulated microgravity conditions, a significant reduction in myostatin expression in OP patients (116.2 ± 14.4) was observed, while higher levels of myostatin expression in both CTRL (145.2 ± 13.5) and OA patients (176.1 ± 16.8) were found (OA vs OP, $p<0.05$). Note that a significant statistical difference was reported between the two CTRL groups ($p<0.0001$), the two OA groups ($p<0.0001$), and the two OP groups ($p<0.0001$). For each patient, the experiment was conducted in triplicate (n=27/group).

Discussion

Life on Earth developed under the influence of normal gravity (1 *g*), therefore organisms are constantly subject to contact forces that provide a series of mechanical stimulations essential for the function of many physiological systems. The lack of normal gravity and the consequent loss of mechanical stimulation of cells and tissues are essential characteristics of space, which is therefore considered a hostile environment [34].

Spaceflights of short and long duration are known to induce a series of physiological and environmental stresses, with a consequent impact on normal physiological processes, such as the functional integrity of muscles and bone mass. For all living organisms, including humans, simulated microgravity exposure can lead to changes in evident muscle atrophy and bone loss [35]. In addition, many of changes observed in astronauts during spaceflights resemble the onset and progression of typical aging diseases, such as osteoporosis, arthrosis and sarcopenia. For this reason, the musculoskeletal system has been one of the main focuses of simulated microgravity research in the last 10 years.

While the main degenerative effects caused by short-term spaceflights are known, the long-term effects are not well understood. It has been hypothesized that in the long-term, simulated microgravity may also affect normal tissue regenerative growth and repair, a process dependent on the proliferation and differentiation of tissue-specific adult stem cells,

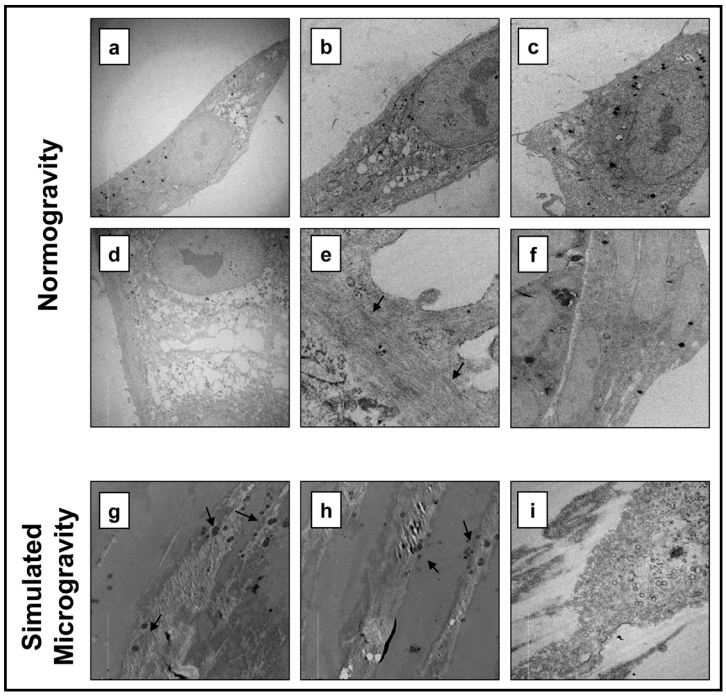

Fig. 5. Ultrastructural analysis of satellite cells in normo- and simulated microgravity conditions. Satellite cells without degeneration sign in normogravity (a,b); small mononuclear cell (c); myotube in formation (d); cytoplasm of a myotube characterized by numerous filaments (arrows), such as neoformation of sarcomeric structures (e); multinucleated myotube (f). In simulated microgravity, myotubes (g,h) and mononuclear cells (i) with evident signs of cell degeneration: in particular, myotubes showed numerous lipofuscin granules with electrondense cytoplasm (arrows) and without the common cell organelles.

which are progenitors of mature terminally differentiated cells in most tissues [36]. The cell culture experiments have been conducted in space using various types of cells, including somatic stem cells, embryonic stem cells and cell culture lines, to determine the influence of simulated microgravity on cell function. These experiments showed that simulated microgravity induces harmful effects on cell function, including inhibition of osteoblasts differentiation [4, 37], reduced osteoblasts numbers [38], atrophy of skeletal muscle cells [39], impaired activation of immune cells [40] and abnormal formation of chondrocyte [41], thus showing the impact of microgravity on various physiological systems.

Based on this evidence, we hypothesized that primary cultures of human satellite cells represent a useful scientific model to identify biomolecular processes involved in muscle mass loss related to the alteration of the normal mechanical load.

For this aim, we performed morphological and immunohistochemical studies on 27 muscle biopsies taken from control (CTRL), osteoporotic (OP) and osteoarthritic (OA) patients, underwent hip arthroplasty. For each patient, we set up primary satellite cell cultures subjected to normo- and simulated microgravity regimens. Morphological analysis showed that satellite cell cultures of the different experimental groups had peculiar morphological characteristics. In normogravity conditions, samples derived from CTRL and OA patients showed a similar appearance, characterized by the presence of numerous myotubes and rare single satellite cells. In contrast, primary cultures derived from OP patients consisted mainly of single satellite cells. These observations agree with our previous studies carried out on biopsy findings, in which satellite cells of OP patients were only rarely active and involved in muscle regeneration [18]. Morphological analysis also showed that simulated microgravity was able to induce important cellular changes. In fact, we observed a significant increase in myotubes in all experimental groups. However, already at the level of optical microscopy, it was possible to observe some signs of cell degeneration, such as cytoplasmic vacuolization.

In order to identify the main molecules involved in the phenomena of degeneration/regeneration of muscle tissue related to the alteration of mechanical load, we assessed BMP-2 expression through immunocytochemical reactions. Noteworthy, in a previous study we demonstrated that the activity of satellite cells was influenced by BMPs expression (BMP-2 and BMP-4), which play a key role in the control of muscle mass and regeneration [18]. Immunocytochemical analysis showed that simulated microgravity regimen was able to influ-

ence primary cultures of satellite cells not only morphologically but also from a molecular point of view. In fact, in normogravity conditions we observed a greater BMP-2 expression in the CTRL group as compared to the other experimental groups. On the contrary, exposure to simulated microgravity led to a significant increase in BMP-2 expression in all experimental conditions. Surprisingly, simulated microgravity condition was able to induce BMP-2 expression in satellite cells that showed an impairment in BMP-2 expression in normogravity regime (OP group).

In a previous study, we hypothesized that the quality of muscle tissue depends on the balance between BMPs signaling and myostatin signaling [22]. For this reason, we have studied the functionality of satellite cells also evaluating myostatin expression, a myokine that influences muscle mass through negative regulation of myogenesis [23]. Specifically, we observed an age-dependent myostatin expression in cell cultures subjected to the normogravity regime. In fact, this was more expressed in OP and OA patients than in the control group. In addition, we found the highest levels of myostatin expression in satellite cells derived from OP patients. It is important to underline that these results also conformed to those obtained on biopsy findings, in which myostatin was mainly associated with the atrophic areas of OP patients [22]. Our results also demonstrate that simulated microgravity was able to induce considerable variations in myostatin expression. Respect to BMP-2, where simulated microgravity was able to stimulate its expression in all experimental conditions, myostatin showed a group-dependent variation. In particular, after 110h of exposure to simulated microgravity, we observed a significant reduction in myostatin expression in OP patients, while expression levels were higher in both CTRL and OA patients.

Finally, we performed an ultrastructural evaluation by using Transmission Electron Microscopy (TEM) to identify satellite cells based on their structural characteristics and to confirm results previously obtained. TEM analysis showed that cell cultures maintained in normogravity were characterized by viable and well-differentiated cells, while exposure to simulated microgravity induced degeneration and cell death phenomena (such as presence of lipofuscin granules, electrondense cytoplasm and dystrophic calcifications).

The degeneration of satellite cells and their consequent inability to replace and repair damaged muscle fibers appears to be closely related to the mechanisms involved in the decline of muscle mass during sarcopenia. The main cause of reduced satellite cell function may be alteration of systemic factors that regulate cellular activity and differentiation. For example, numerous members of the TGF-β family, including BMPs and myostatin, have been shown to play important roles in regulating muscle growth and atrophy. As supported by our results, exposure to simulated microgravity could amplify these mechanisms, inducing significant reductions in the size, volume and strength of skeletal muscles and thus leading to further muscle atrophy and weakening. Simulated microgravity induces atrophy in much faster times than it does, for example, in aging atrophy. Therefore, it would be important to understand why satellite cells, which normally should keep muscles in a trophic state, are unable to respond and perform their role effectively, even more so when exposed to a simulated microgravity regimen.

Taken together, the morphological and molecular characterization of muscle biopsies collected in this study allowed us to confirm the importance of BMP-2 and myostatin pathways in physio-pathogenesis of muscle tissue. It is important to note how satellite cells respond to the simulated microgravity condition in a very similar way to what occurs *in vivo* following an injury and/or organ damage. In fact, simulated microgravity regime induces the formation of myotubes by increasing the synthesis of BMP-2. Furthermore, in cell cultures characterized by a low ability to form myotubes, simulated microgravity can stimulate, at least in initial stages, cellular regeneration by lowering myostatin expression levels (mainly in OP patients).

Therefore, our data allow us to hypothesize a possible molecular mechanism in which satellite cells response to simulated microgravity: in early stages, satellite cells respond to simulated microgravity by a) forming new myotubes and b) increasing BMP-2 expression

and c) reducing myostatin expression. As the simulated microgravity condition persists, myostatin expression increases and cells undergo degeneration phenomena.

Limits of the study

The main limit of this study is represented by the controls used to correlated data of OP patients, especially for the different average age between OP, or OA, and CTRL. Unfortunately, it is very difficult to collect muscle biopsies of patients aged >60 years without OP or OA who underwent hip arthroplasty for high-energy hip fractures. However, the results demonstrated that OA patients were good surrogate controls in this study, showing several similarities with younger patients.

Conclusion

Experiments here reported have shown how the study of primary satellite cell cultures can represents an efficient tool for investigating effects of simulated microgravity on humans. Results obtained can lay the foundations for the characterization of processes involved in the cellular response to simulated microgravity and for the identification of specific therapeutic targets useful for the prevention/cure of muscle pathological conditions related to alteration of mechanical load (e.g. sarcopenia).

Acknowledgements

Author Contributions

UT developed the hypotheses and designed the experimental plan. MS, IC and MM performed and analyzed experiments and contributed to the interpretation of the data. MP, RI and EG participated in the study and cases selection. IC, GD and VT wrote and edited the manuscript. UT and MS assisted in drafts and final version of the manuscript. All authors read and approved the final manuscript.

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Statement of Ethics

All sampling and experiments described in the present study were performed in agreement with independent ethical committee of "Policlinico Tor Vergata" (approval reference number 85/12). All experimental procedures were carried out according to The Code of Ethics of the World Medical Association (Declaration of Helsinki). Informed consent was obtained from all patients prior to surgery. Specimens were handled and carried out in accordance with the approved guidelines.

Disclosure Statement

The authors declare that they have no competing interests.

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