Irisin prevents microgravity-induced impairment of osteoblast differentiation *in vitro* during the space flight CRS-14 mission

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Running title Irisin boosts osteoblasts in microgravity

ABBREVIATIONS

aMEM Minimum Essential Medium Eagle, alpha modification

EC Endothelial Cell

FCS Fetal Calf Serum

HU hindlimb suspended mice

ISS International Space Station

OB Osteoblast

OC Osteoclast

PBS Phosphate Buffer Saline

ABSTRACT

Understanding molecular mechanisms responsible for bone cells unbalance in microgravity would allow the development of better countermeasures for astronauts, and eventually advancing terrestrial osteoporosis treatments.

We conduct a unique investigation by using a controlled 3D in vitro cell model to mimic the bone microenvironment in microgravity aboard the SpaceX Dragon cargo ferry to the ISS. Osteoblasts, osteoclasts and endothelial cells, seeded on Skelite™ discs, were cultured w/ or w/o rec-Irisin and exposed to 14 days of microgravity in the eOsteo hardware. Gene expression analysis was assessed, and results were compared to ground controls treated within identical payloads.

Our results show that the microgravity-induced downregulation of mRNA levels of genes encoding for osteoblast key transcription factors (*Atf4* -75%, $p< 0.01$; *RunX2* -87%, $p< 0.001$, *Osterix* -95%, $p< 0.05$ Vs ground) and proteins (*Collagen I* -84%, $p< 0.05$; *Osteoprotegerin* -94%, $p< 0.05$) were prevented by irisin. Despite it was not effective in preventing Trap and Cathepsin K mRNA increase, irisin induced a 2.8-fold increase of Osteoprotegerin ($p< 0.05$) that might act for reducing osteoclastogenesis in microgravity.

Our results provide evidence that irisin supports OB differentiation and activity in microgravity and it might represent a countermeasure to prevent bone loss in astronauts.

Key Words: irisin, osteoblasts, microgravity, ISS

INTRODUCTION

Mechanical forces are central in maintaining skeletal integrity. During both short- and long-duration space missions, astronauts severely lose bone mass; bone thins, weakens and the risk of fractures becomes high upon entering on Earth's gravity. In loading condition, bone health and integrity are maintained by a well-orchestrated activity between bone-resorbing osteoclasts (OC) and bone-forming (OB). On the contrary, as demonstrated in different in vitro studies, both in space and simulated microgravity on earth, the resorption exceeds bone formation due to both enhanced OC activity and reduced OB differentiation (1). Our previous *in vitro* study in space, during FOTON-M3 European Space Agency (ESA) Mission in September 2007, indicated, for the first time, that OC and their precursors are direct targets of microgravity and mechanical forces as demonstrated by the increased expression profile of genes involved in their formation and activity (2). Concerning the reduction of bone deposition, in addition to the impairment of OB formation and differentiation, other findings both in space, aboard STS-56 shuttle flight and Foton M3 satellite, and in simulated microgravity (3-4) have demonstrated osteoblastic impairment due to disruption of cytoskeletal organization, thereby contributing to bone formation decrease in the absence of gravity.

Intriguingly, the bone cells interact with other cells for controlling the bone remodeling and homeostasis, including the endothelial cells. Indeed, bone-endothelial cell crosstalk occurs directly through intercellular junctions and indirectly by means of mutual paracrine factors. Endothelial cells release osteogenic factors while *vice versa* bone cells are source of angiogenic ones (5-6).

Importantly, bone-endothelial cell coupling is altered in simulated microgravity (7). In unloading conditions endothelial cells grow less, enhance the secretion of factors inhibiting OB proliferation and activity (6), and increase the release of osteoclastogenic cytokines (8-9) thereby supporting endothelium contribution in unloading-induced bone loss.

Notably, other cells are crucial for bone health, these are skeletal muscle fibers. Although bone-muscle anatomical interaction has always been known, we today recognize that muscle acts as endocrine organ targeting bone through circulating myokines (10). Likewise, bone exerts an endocrine control on muscle through the release of circulating cytokines. Bone can adapt its mass and architecture consistent with changes in muscle-applied mechanical loading, so that a decrease in muscle function gives rise to a reduction in the bone loading leading to bone loss. This occurs when sarcopenia and osteoporosis develop in parallel.

It has been proved that in simulated microgravity alterations in muscle take place before those in bone, and changes in osteoclastogenic cytokine release following paralysis happens before the resorption begins. It has been widely recognized that physical exercise displays beneficial effect on bone and can prevent bone and muscle loss. At present we know that the molecular mediator that

couples the beneficial effect on bone and muscle, which has remained unknown for a long time, is irisin. This molecule, discovered in 2012, is produced by skeletal muscle after physical activity, both in mice and in humans, and it was originally described as capable of promoting the trans-differentiation of white adipose tissue into the brown type (11). However, our studies revealed that the main target tissue of irisin is bone as intermittent administration of irisin in healthy mice, used at lower concentration than that active on adipose tissue, displays anabolic effect on bone through the stimulation of osteoblast formation and activity (12). Subsequently, we proved that irisin administration prevents the development of disuse-induced osteoporosis and muscular atrophy in hindlimb suspended mice (HU), a murine model which mimics adverse effects on musculoskeletal system caused by prolonged bed rest, physical immobility and microgravity exposure (13). These findings indicated that irisin can both prevent and cure, Osteoporosis and Muscular Atrophy at the same time, two conditions that affect astronauts during space mission. Therefore, in this study by using the e-Osteo hardware equipped with fluid pathway bioreactors, we assessed a controlled 3D in vitro cell coculture model, consisting of OB, OC and EC cells seeded on Skelite™ discs, to mimic the bone *in vivo*. With this *in vitro* experimental model, we studied for the first time irisin effect on bone cell behavior aboard the SpaceX Dragon cargo ferry to the International Space Station (ISS) during the space flight CRS-14 mission. Our results showed that OB differentiation is critically affected by microgravity and, for the first time, provided evidence that irisin improves OB activity in space, thereby suggesting that irisin might be in the future a countermeasure to prevent bone loss in astronauts.

MATERIALS AND METHODS

Cell culture preparation

Primary murine osteoclast (OC) precursors were obtained from bone marrow cultures from 8-wk-old male C57BL6 mice (Charles River Laboratories, Wilmington, MA, USA). The proximal and distal ends of freshly harvested mice femurs were excised, and cells were collected by flushing the medullar cavity with ice-cold α-minimum essential medium (αMEM) through a 25-gauge needle. The whole marrow was plated in Petri dishes and after 24 h, non-adherent cells were collected and cultured for an additional 4 days with αMEM + 10% fetal calf serum (FCS) in the presence of 10 ng/ml of macrophage colony stimulating factor (MCSF; R&D Systems, Minneapolis, MN, USA). Endothelial cells (EC)-EOMA (ATCC® CRL-2586™) and murine Osteoblasts (OB)-MC3T3E1 (ATCC® CRL2593) (LGC, Middlesex, UK) were cultured in αMEM + 10% fetal calf serum (FCS). Four days before launch, three different types of 3D *in vitro* cell cocultures (OB/OC,

OB/EC, OB/EC/OC) were set up by seeding OBs ($0.4x10^6/cm^2$), EC ($0.2x10^6/cm^2$) and OC ($0.5x10^6/cm^2$) on bone-like synthetic scaffolds (Skelite™, Millenium Biologix, Kingston, ON, Canada) in 24-multi-well plates in αMEM + 10% FBS + MCSF 10 ng/ml.

eOSTEO hardware description

The fully automated eOSTEO flight Hardware (Calm Technologies, Ontario, Canada) (Fig. 1) consists of three independent trays each operating at 37±0.5°C at all times, including during transport and launch. Each tray houses a closed fluid pathway network, where three cell culture bioreactors receive fluids from syringe reservoirs via a multiple valve system. Each bioreactor houses a holder for 5 scaffolds. The hardware was programmed to give regular media changes to the cells in the bioreactors with a medium refresh rate of 1ml/min. The three trays in the eOsteo hardware are comprised in an EXPRESS mid-deck locker replacement payload. The locker system provides a common power, data/communications, and thermal interface (avionics air) for the trays, and a means of securing the trays for launch and storage. *In Vitro Bone* experiment required a total of six trays (two for each experiment), divided into two e-Osteo-ISS lockers.

Priming and loading the eOSTEO flight system multi-syringe fluid pathway

We primed and loaded two identical eOsteo trays, one with control medium and one with recombinant Irisin (rec-irisin) in the medium. All the syringes in both trays were filled with the appropriate fluids: three syringes with αMEM/10% FCS + L-ascorbic acid-2-phosphate 50 µg/ml + β-Glycerophosphate 10^{-2}M + 5 ng/ml MCSF + 3 ng/ml RANK-L ± recombinant (rec)-irisin 100 ng/ml, one syringe with phosphate buffered saline (PBS) and one syringe was loaded with RNAlater (Qiagen, Germantown, MD, USA) (Fig. 1).The αMEM was modified by the addition of sodium chloride (700 mg/L) and sodium bicarbonate (350 mg/L) and buffered by HEPES (25 mM). After priming the syringes, the eOSTEO bioreactors were loaded with cells plated on skelite scaffolds, and eventually the remaining fluid lines and modules were completely filled before closing all valves and committing to ESA engineers 24 hours before lunch (L-24 hrs). After 14 days in microgravity, before the cells entered the earth's atmosphere and experienced gravity conditions, PBS wash was performed followed by fixation with RNAlater. Soon after fixation, the temperature in the eOsteo hardware was maintained between 2-10°C. After landing, skelite scaffolds were recovered from the bioreactors, and the cells were subjected to RNA extraction. A duplicate control of *In Vitro Bone* experiment was performed in another payload containing the two Trays (± rec-irisin 100 ng/ml) assembled in identical preflight conditions and kept on the ground for the same duration of the flight (ground reference experiment).

Real Time-PCR

Total RNA was extracted using spin columns (RNeasy, Qiagen) according to the manufacturer's instructions. DNase I treatment was performed to remove genomic DNA contamination (Qiagen) and RNA integrity was assessed on agarose gels. Reverse transcription was performed using iScript Reverse Transcription Supermix (Bio-Rad). The resulting cDNA (20 ng) was subjected to quantitative PCR (qPCR) using the SsoFast EvaGreen Supermix (Bio-Rad) on an Bio-Rad CFX96 Real-Time System (Bio-Rad) for 40 cycles (denaturation 95°C for 5 s; annealing/extension 60°C 10 s) after an initial 30 sec step for enzyme activation at 95°C. To confirm the specificity of amplification products, melting curve was performed between 65-96°C, with 0.5°C incrementing every 10 sec. Primers were designed by using Primer Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). We chose Gapdh as housekeeping gene because it is stably expressed. Primer sequences: *Gapdh* (S-acaccagtagactccacgaca, AS-acggcaaattcaacggcacag); *Atf4* (S-gcctgactctgctgcttacattac, AS-cacgggaaccacctggagaag); *RunX2* (S-accagcagcactccatatctctac, AS-tccgtcagcgtcaacaccatc; *Osterix* (S-agttcacctgcctgctctgttc, AS-gcggctgattggcttcttcttc); *Opg* (S-gaccactcttatacggacag, AS-ctcacactcacacactcg); *Cathepsin K* (S-gggtgttcaagtttctgctgctac, AS-actgcttctggtgagtcttcttcc); *Trap* (S-gcagcagccaaggaggactac, AS-agcacatagcccacaccgttc).

All primers span an exon-exon junction. Each transcript was assayed in triplicate and quantitative measures were obtained using the ΔΔCT method and expressed as a fold change compared to control.

Statistics

Data were analyzed using ANOVA, followed by Tukey's post hoc analysis to compare the groups. Values were considered statistically significant at $p<0.05$. Results were obtained from 5 biological replicates, each one with 3 technical replicates. All quantitative data are presented as mean ± SEM.

RESULTS

By Real time PCR we evaluated the gene expression levels of the master osteoblastic and osteoclastic markers in the three 3D co-culture systems, OC/OB, OB/EC, and OC/OB/EC, seeded on Skelite discs and integrated in the bioreactors in both ground and flight experiments. We first examined the expression of osteoblastic transcription factor genes, such as the Activating transcription factor 4 (*Atf4*), Runt-related transcription factor 2 (*RunX2*) and Transcription factor Sp7 (*Osterix*) in all the coculture systems. Figure 2 shows that in OC/OB/EC co-cultures all the

transcription factors were severely reduced by microgravity (*Atf4* -75%, $p<0.01$; *RunX2* -87%, $p<0.001$; *Osterix* -95%, $p<0.05$ Vs ground controls), whereas treatment with rec-irisin (100 ng/ml) fully prevented *Atf4* and *RunX2* mRNA decrease (Fig. 2a-b). In particular, the irisin-dependent increase of *Atf4* gene levels in flight conditions was about tenfold higher compared to the absence of the myokine ($p<0.05$ Vs flight Untreated) and almost twofold greater than the irisin-induced increase of *Atf4* gene levels on ground (+40%, $p<0.05$ Vs ground Untreated) (Fig 2a). In space, irisin significantly enhanced *Runx2* gene expression ($p<0.05$ Vs flight Untreated) which levels were quite similar to baseline expression on ground, whereas in this latter condition *Runx2* expression is not affected by irisin. Moreover, in flight samples, *Osterix* mRNA levels, although not fully restored to ground control, were 5-fold increased by rec-irisin treatment ($p<0.001$) Vs untreated cells (Fig. 2c).

We next assessed the mRNA levels of the main proteins, *Collagen I* and Osteoprotegerin (*Opg*), produced by OBs in OC/OB/EC 3D cocultures. We demonstrated that *Collagen I* was negatively affected by microgravity (-84%; $p<0.05$ Vs ground control), and the presence of rec-irisin in the medium of OC/OB/EC co-cultures during flight induced a 4-fold increase of Collagen I mRNA ($p<0.001$) (Fig. 3a). Moreover, we showed that *Opg* mRNA expression was severely downregulated by microgravity (-94%; $p<0.05$ Vs ground control), whereas its levels were preserved at the same level of control cells (ground Untreated) by rec-irisin treatment. Interestingly, rec-irisin treatment induced a 2.6-fold increase of *Opg* expression in ground control cocultures ($p<0.05$ Vs ground Untreated) (Fig. 3b).

Then we evaluated the expression of osteoclastic master genes, *cathepsin K* and resistant acid tartrate phosphatase (*Trap*), and the effect of irisin on the gene levels of the two marker enzymes of osteoclast differentiation.

Expectedly, OC differentiation was enhanced in microgravity. Figure 4 shows that in OB/OC co-cultures the expression of both osteoclastic markers increased compared to ground levels as demonstrated by 1.5-fold increase of *Cathepsin K* and by 5-fold increase of *Trap* mRNA ($p<0.05$) (Fig. 4a-b). We further found that the presence of rec-irisin did not modify their expressions. In parallel, we found a 3-fold Irisin-induced increase of *Opg* expression ($p<0.05$) (Fig. 4c) suggesting that this effect might compensate for controlling osteoclast activity in microgravity.

DISCUSSION

Here we show that the myokine irisin, widely known for its osteoanabolic activity (12), prevents the downregulation of some relevant transcription factors and proteins responsible for osteoblast

functions in an *in vitro* 3D coculture system on board of the ISS during the space flight CRS-14 mission.

Due to the challenges posed by long space flights, such as the future mission to Mars, the effects of microgravity on bone metabolism have been a broad field of study in recent years. Thus far, the scientific community is making considerable efforts in this field aiming to prevent one of the most critical biomedical problem that astronauts will encounter: the sharply loss of bone mass.

Although several results have shown that microgravity has a negative impact on bone metabolism mainly due to hyperactivation of bone resorption, this effect was not always sufficient to explain the dramatic decrease in bone mass observed in astronauts, which would also imply hypo-functionality of the osteoblasts (14). Nevertheless, microgravity also causes negative effects on muscle tissue in which it determines significant loss of mass (15) confirming that bone and muscle damage proceed in parallel, and that the two tissues are functionally and molecularly linked, such as happens on Earth when osteoporosis and muscle atrophy develop simultaneously.

In this regard, we have previously shown that the myokine irisin prevents and restores bone loss and muscle atrophy in HU mice, a murine model of both simulated weightlessness and disuse-induced osteoporosis and muscle atrophy (13). Therefore, in this study we sought to determine whether the microgravity-induced dysregulation of bone forming cell activity could be prevented by the presence of irisin in the culture medium. Moreover, the rationale of our study stems from the knowledge that ensuring astronauts perform regular, daily physical activity is currently the major countermeasure to prevent their bone loss during flight mission (16). Therefore, we hypothesized that irisin, a key molecule produced by the skeletal muscle during physical activity, could positively affect bone forming cells in an *in vitro* bone environmental model as mimetic-exercise molecule in space flight.

Our findings showed that the microgravity-induced mRNA downregulated expression of *Atf4*, a key transcription factor for proliferation, differentiation and survival of osteoblasts and osteocyte (17, 18), is fully prevented by the presence of irisin. The most significant result was found in the 3D three cell type coculture (OB/EC/OC) that most closely mimics the bone microenvironment. We not only proved the strong reduction of *Atf4* in space, but also that the irisin-mediated increase of Atf4 in flight is significantly higher than the increase observed in the ground reference experiment. This result is also in line with our previous studies in which we demonstrated that *Atf4* is the main target gene modulated by irisin in osteoblasts (12, 19) and more recently in osteocytes (20). Importantly, in flight irisin also up-regulated significantly the mRNA expression of *RunX2*, essential transcription factor for bone development and osteoblast differentiation which also forms a functional complex with *Atf4*, critical in the regulation of bone formation (21). We revealed that the

irisin-induced rise in *Runx2* gene expression reached the basal value, and *Osterix* (22) levels were also increased by irisin, although did not return to the ground control expression. These results suggest that irisin in microgravity, by increasing three of most relevant transcription factors for osteoblastogenesis, contributes to maintain in flight the OB phenotype and preserves these cells from the significant weightlessness-induced decline of their characteristics.

We also investigated the mRNA expression levels of two critical proteins produced by osteoblasts. Firstly, we analyzed *Collagen I*, the most abundant protein secreted by osteoblasts in the matrix, whose expression is regulated by *RunX2* (23), and thus index of proper osteoblastic activity. Next, we evaluated *Opg*, the well-known anti-osteoclastogenic cytokine, expressed by osteoblasts to control bone resorption for maintaining bone mass under physiological range (24). The treatment with irisin prevented the dramatic downregulation of their mRNA levels caused by microgravity. Regarding *Opg*, our result was also in agreement with our previous *in vivo* finding showing that irisin, intermittently injected once a week for 28 days, upregulated *Opg* expression in cortical bone of HU mice (13). Interestingly, in ground control OB/EC/OC coculture, we found that *Opg* mRNA levels were significantly increased by treatment with irisin in comparison with untreated coculture. This result shows for first time a direct action of irisin on *Opg* mRNA expression in a 3D system of bone and endothelial cells *in vitro*.

Regarding osteoclasts, we evaluated the effect of irisin on the expression of two key enzymes, cathepsin K (25-26) and Trap (27-28). In agreement with our previous findings (2), we found in OB/OC cocultures that both enzymes were significantly up regulated after microgravity exposure compared to ground controls. However, treatment with irisin did not modify their mRNA expression levels. Noteworthy, although irisin showed no efficacy in directly controlling osteoclastic markers, it significantly upregulated *Opg* mRNA expression, thus suggesting that irisin could inhibit the net process of osteoclast differentiation and bone resorption by modulating this anti-osteoclastogenic cytokine.

In conclusion, our data highlight the effect of irisin in controlling the bone remodeling process severely affected in the absence of gravity. Irisin is a molecule that acts as a balance between the reduced osteo-deposition activity and the high bone resorption of osteoclasts. This effect is mainly exerted directly favoring the differentiation and activity of osteo-forming cells and counteracting, through the modulation of Opg, the hyper-activity of osteoclasts. These results suggest that irisin could be in the future a valid countermeasure for the loss of bone mass induced by microgravity during space missions. Current countermeasures for loss of bone mass and density in astronauts, which include intense exercise, controlled diet and vitamin D supplementation while in orbit, cannot alone reduce the risks to develop osteoporosis. Moreover, once back on Earth, the negative

impact on the astronauts' skeleton is only partially reversed. Indeed, it has been observed that the newly synthesized bone tissue is more porous and with reduced mineral density compared to the bone status before the space expedition (29). Considering these results, it has been assumed that long-term exploration, such as that of Mars, would have more serious consequences for the health of their bones. Therefore, the development of new pharmacological treatment could be of strategic importance as a preventive or curative therapy.

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AUTHOR CONTRIBUTIONS

S. Colucci and M. Grano designed research; G. Colaianni, G. Brunetti, and G. Mori analyzed data; S. Colucci, G. Colaianni, G. Brunetti, G. Mori, and M. Grano performed research; F. Ferrante, G. Mascetti, S. Colucci and M. Grano discussed and critically interpreted data; S. Colucci and M. Grano wrote the paper.

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FIGURE LEGENDS

Figure 1 Photo showing the fluid pathway housed in the fully automated eOSTEO flight Hardware (Calm Technologies, Ontario, Canada), which consists of three independent trays each operating at a defined temperature. In each fluid pathway, three cell culture bioreactors (bottom left) receive fluids from syringe reservoirs via a multiple valve system. The hardware was programmed to give regular media changes to the cells in the bioreactors and, after 14 days in microgravity before the cells entered the earth's atmosphere and experienced gravity conditions, PBS wash was performed followed by fixation with RNAlater. In the inset (black arrow) is shown a representative drawing of

the three bioreactors housing 3D *in vitro* cell cocultures (OB/OC, OB/EC, OB/EC/OC) seeded on bone-like synthetic scaffolds (Skelite™).

Figure 2 Quantitative PCR (qPCR) showing modulation of mRNA expression levels of (**a**) *Atf4*, (**b**) *RunX2*, and (**c**) *Osterix* assayed on 3D *in vitro* cell cocultures (OB/EC/OC) cultured for 14 day in ground conditions or in flight (microgravity) with a-MEM modified/10% FCS supplemented with bone cell differentiation medium (L-ascorbic acid-2-phosphate 50 µg/ml + b-Glycerophosphate 10^{-2}M + 5 ng/ml MCSF + 3 ng/ml RANK-L) ± recombinant (rec)-irisin 100 ng/ml. Gene expression was normalized to *Gapdh* and plotted as fold-increase from the untreated (ground) samples (n=5 independent scaffold discs). ANOVA test, followed by Tukey's post hoc analysis, were used to compare treatments. Data are presented as mean +/− SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Figure 3 Quantitative PCR (qPCR) showing modulation of mRNA expression levels of (**a**) *Collagen I*, and (**b**) *Opg* assayed on 3D *in vitro* cell cocultures (OB/EC/OC) cultured for 14 day in ground conditions or in flight (microgravity) with a-MEM modified/10% FCS supplemented with bone cell differentiation medium ± recombinant (rec)-irisin 100 ng/ml. Gene expression was normalized to *Gapdh* and plotted as fold-increase from the untreated (ground) samples (n=5 independent scaffold discs). ANOVA test, followed by Tukey's post hoc analysis, were used to compare treatments. Data are presented as mean +/− SEM. *$p < 0.05$, ***$p < 0.001$.

Figure 4 Quantitative PCR (qPCR) showing modulation of mRNA expression levels of (**a**) *Cathepsin K I*, (**b**) *Trap,* and (**c**) *Opg* assayed on 3D *in vitro* cell cocultures (OB/OC) cultured for 14 day in ground conditions or in flight (microgravity) with a-MEM modified/10% FCS supplemented with bone cell differentiation medium ± recombinant (rec)-irisin 100 ng/ml. Gene expression was normalized to *Gapdh* and plotted as fold-increase from the untreated (ground) samples (n=5 independent scaffold discs). ANOVA test, followed by Tukey's post hoc analysis, were used to compare treatments. Data are presented as mean +/− SEM. *$p < 0.05$.