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Exposure to microgravity for 30 days onboard Bion M1 caused muscle atrophy and impaired regeneration in murine femoral *Quadriceps*

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ABSTRACT

Mechanical unloading in microgravity during spaceflight is known to cause muscular atrophy, changes in muscle fiber composition, gene expression, and reduction in regenerative muscle growth. Although some limited data exists for long-term effects of microgravity in human muscle, these processes have mostly been studied in rodents for short periods of time. Here we report on how long-term (30-day long) mechanical unloading in microgravity affects murine muscles of the femoral *Quadriceps* group. To conduct these studies we used muscle tissue from 6 microgravity mice, in comparison to habitat (7), and vivarium (14) ground control mice from the NASA Biospecimen Sharing Program conducted in collaboration with the Institute for Biomedical Problems of the Russian Academy of Sciences, during the Russian Bion M1 biosatellite mission in 2013. Muscle histomorphology from microgravity specimens showed signs of extensive atrophy and regenerative hypoplasia relative to ground controls. Specifically, we observed a two-fold decrease in the number of myonuclei, compared to vivarium and ground controls, and central location of myonuclei, low density of myofibers in the tissue, and of myofibrils within a fiber, as well as fragmentation and swelling of myofibers. Despite obvious atrophy, muscle regeneration nevertheless appeared to have continued after 30 days in microgravity as evidenced by thin and short newly formed myofibers. Many of them, however, showed evidence of apoptotic cells and myofibril degradation, suggesting that long-term unloading in microgravity may affect late stages of myofiber differentiation. Ground asynchronous and vivarium control animals demonstrated normal, well-developed tissue structure with sufficient blood and nerve supply and evidence of regenerative formation of new myofibers free of apoptotic nuclei. Regenerative activity of satellite cells in muscles was observed both in microgravity and ground control groups, using Pax7 and Myogenin immunolocalization, as well as *Myogenin* expression analysis. In addition, we have detected positive nuclear immunolocalization of c-Jun and c-Myc proteins indicating their sensitivity to changes in gravitational loading in a given model. In summary, long-term spaceflight in microgravity caused significant atrophy and degeneration of the femoral *Quadriceps* muscle group, and it may interfere with muscle regenerative processes by inducing apoptosis in newly-formed myofibrils during their differentiation phase.

1. Introduction

Changes in the musculoskeletal system under the influence of microgravity are among the main topics of gravitational biology and medicine. It is well established that gravitational unloading of mammalian and human skeletal muscles leads to muscular atrophy, and hinders muscular regeneration (Grigor'ev et al., 2004; Martin et al., 1988; Schultz et al., 1994; Shenkman et al., 2010). Laboratory experiments using various models of muscular unloading have provided detailed fundamental information regarding morphological, biochemical and molecular processes that lead to degeneration and atrophy of muscle fibers (Baldwin et al., 2013; Sandri, 2013). In brief, the current understanding of this process is that the physical signal of mechanical unloading is translated into a molecular signaling process that leads to degradation of fibers on biochemical level, followed by a morphological manifestation of the process as significant muscle mass loss (Baldwin et al., 2013; Chopard et al., 2009; Morey-Holton et al., 2005). In addition, the possibility of compensatory processes aiming to maintain viability and function of muscle tissue in animals undergoing prolonged gravitational unloading has been also been reported (Sandonà et al.,

Abbreviations: PBS, phosphate buffered saline; PCR, polymerase chain reaction

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2012).

However, flight experiments studying the processes of muscular hypotrophy and atrophy have mostly been relatively short-term (up to 2 weeks under microgravity) (Desplanches et al., 1990; Kraemer et al., 2000; Ohira et al., 2002, 1992; Schuenke et al., 2009; Staron et al., 1998). Pooled data indicates that short-term flights drastically affect muscular system by inducting its partial degeneration and transforming slow twitch fibers into fast twitch ones. These effects are also apparent on molecular level, in particular in terms of mRNA expression of multiple genes involved in growth and differentiation of muscle fibers (Allen et al., 2009). Much less data has been acquired for animals exposed to long-term flights. Despite limitations related to low numbers, it has been reported that soleus muscles in mice kept on the International Space Station for 91 days of the MDS (mice drawer system) experiment demonstrated the same amount of atrophy that occurs during 20 days of flight. Genes encoding atrophy-related ubiquitin ligases were up-regulated in these muscles, as well as in *extensor digitorum longus* (Sandonà et al., 2012).

The work we present here investigates mouse muscles from *Quadriceps* group, obtained from the hip region adjacent to the femoral head. The pelvic musculoskeletal complex in mice is known to be highly loaded under normal ambulation conditions at 1 g, and thus very sensitive to gravitational unloading, making it a valuable model to study musculoskeletal degeneration in spaceflight. Simultaneously with degenerative processes we were also able to study muscle regeneration; both processes have been studied for the first time in microgravity for this particular muscle group.

Muscle regeneration can be induced in response to injury, ageing and disease, over-exercise and trauma (Brooks and Myburgh, 2014; Masiero et al., 2009). Cellular sources and triggering molecular mechanisms of muscular regeneration have also been studied (Kawano et al., 2008; Shenkman et al., 2010). Specifically it has been shown that injury to muscle fibers results in a cascade of events, leading to the escape of satellite cells from the basement membranes, leading to proliferation and myoblast development, and later differentiation and morphogenesis of new muscle fibers (Yablonka-Reuveni et al., 2008). It is also known that artificial unloading of muscles inhibits muscle fiber regeneration (Darr and Schultz, 1989; Mozdziak et al., 1998). Regeneration in microgravity thus may be inhibited and insufficient to compensate for the full extent of muscle damage that is induced in space (Matsuba et al., 2009). This regenerative deficit has been attributed to the suppression of satellite cells' activation and their interaction with macrophages, which are also inhibited by microgravity (Kohno et al., 2012). However, it is still unclear which stages of muscle regeneration are blocked by gravitational unloading, and via what molecular mechanisms.

In this study we aimed to further elucidate the processes of muscle degeneration and regeneration in a highly loading-sensitive murine muscles (*Quadriceps* group) during a long-term spaceflight (30 days) onboard the Bion M1 biosatellite. We did this in existing and regenerating muscle fibers using histo-morphometric analysis, visualization of apoptosis, and immunolocalization of several markers for the initiation of muscle regeneration as well as *Myogenin* gene expression, for evaluating later stages of muscle regeneration.

2. Materials and methods

2.1. Animal handling and groups

The current study was conducted on mice involved in the spaceflight experiment onboard Russian biosatellite Bion M1. Male C57Bl/6N SPF mice 8–9 weeks of age were obtained from the breeding facility of M.M. Shemyakin and Y.A. Ovchinnikov Institute of Bioorganic Chemistry RAS in Pushchino, Russia. Details of pre-flight selection, experiment's bioethics, preparations for flight, as well as conditions in flight and in control are detailed in the reports of the Bion M1 working team (Andreev-Andrievsky et al., 2014; Sychev et al., 2013). Muscles were collected from 6 animals shortly after the landing, 7 animals of an asynchronous spaceflight habitat ground control (kept on Earth in containers and conditions closely mimicking those of spaceflight group) and 14 animals from two vivarium controls (7 from each) that were not exposed to any specific conditions.

2.2. Muscle extraction and histological procedures

Proximomedial portion of *Quadriceps* muscles was extracted surgically at the final stage of the biospecimen program dissection, about 30 to 40min post-euthanasia. Every muscle tissue sample was divided into two parts: one of them was fixed in 4% formaldehyde solution, made on 0.1 M phosphate buffered saline (PBS, pH 7.4), at +4 °C, while the other one (needed for RNA extraction) was immersed in RNA*later*™ (Qiagen) and stored at −20 °C. Formaldehyde-fixed fragments of muscle tissue were then rinsed, dehydrated and embedded in Hystomix using standard histological techniques. Serial tissue sections about 7 μm thick were mounted on slides and stained with hematoxylin-eosin. Tissue morphology was assessed using an Olympus light microscope and a Leica DM5000B epifluorescence microscope with a digital cooled CCD camera; images were obtained using Leica Application Suite software.

2.3. Quantitative analysis of histological sections

Quantitative evaluation of histological samples was performed for 4 randomly selected samples from vivarium controls, 4 samples from ground controls and 5 samples from the flight group. We used two methods to estimate the relative number of myonuclei. In the first case, myonuclei (both from myofibers and satellite cells) were counted manually using the ocular grid within 0.25 mm^2 squares of regular densely packed muscle tissue, avoiding areas with other structures or empty spaces. For each sample 50–70 cross sections from a range of positions along the proximal part of studied muscles were analyzed. In the second case, similar areas of muscle tissue were photographed with a Leica DMRXA2 microscope with Olympus DP70 camera. Images (average of 20 per sample) were analyzed with NIH Image J software by measuring the number of myonuclei relative to both total area (N_c/T_a) and area occupied by eosin-stained myofibers (N_c/F_a). The first approach was used to check for agreement between manual and computer analyses, while the latter was used to correct for empty spaces between the muscle fibers. Data obtained by both methods were transferred to STATISTICA 8.0. for descriptive statistics and group comparisons, which were made using Kruskal–Wallis nonparametric test for multiple independent samples and Dunn's *post hoc* test for multiple comparisons.

2.4. Immunolocalization analysis

Formaldehyde-fixed tissue was rinsed in PBS, immersed into sucrose solutions of rising concentration (5%, 10% and 20% sucrose on 0.1 M PBS, three changes of each) and left into 20% sucrose solution overnight at +4 °C. It was then embedded in Tissue-Tec OCT compound (Leica), frozen and cut into 10 mm thick sections with Leica M1900 cryostat. Sections were mounted on the slides, rinsed with PBS, incubated for 15 min in permeabilizing solution (0.25% Triton X-100, 0.1% Twin-20 on PBS), rinsed, incubated for 60 min with blocking solution (3% bovine serum albumin on PBS). Sections were then incubated for 12 h at +4 °C with primary antibodies dissolved in blocking solution according to the manufacturer's recommendations (see Table 1), rinsed again and incubated for 60 min at room temperature in the dark with secondary antibodies (see Table 1). Finally, they were rinsed, stained with Hoechst 33342 (Leica), and mounted under cover slips in Vectashield medium (Vector). Specificity of the labeling was tested by preparing control slides processed similarly, but incubated in blocking solution instead of primary antibodies (no labeling was seen