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Degenerative Tissue Responses to Space-like Radiation Doses in a Rodent Model of Simulated Microgravity

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Abstract. This study examines acute and degenerative tissue responses to space-like radiation doses in a rodent model of simulated microgravity. We have studied four groups of rats, control (CON), irradiated (IR), irradiated and hindlimb suspended (IR-HLS), and suspended (HLS) that were maintained for two weeks. IR and IR+HLS groups were exposed to five sessions of X-ray irradiation (1.2 Gy each, at 3-4 days intervals). Body weights, soleus muscle weights, and hindlimb bone mineral density (BMD) were measured. Results show that compared to CON animals, IR, HLS, and IR+HLS group reduced the body weight gain significantly. IR-associated growth retardation appeared to be closely linked to acute and transient post-IR 'anorexia' (a decrease in food intake). HLS but not IR induced major changes in the musculoskeletal system, consisting in decreases in soleus muscle mass and bone mineral density of distal femur and proximal tibia. Additional dosimetric studies showed that the effect of IR on weight is detectable at 0.3 Gy X-ray doses, while no threshold dose for the IR-produced decrease in food intake could be observed. This study suggests that space flight-associated anorexia and musculoskeletal degenerative changes may be driven by different, radiation- and microgravity-associated (respectively) mechanisms.

Key words: simulated microgravity, hind limb suspension, radiation effects, bone mineral density, muscle atrophy.

Introduction

Perspectives of future long-term space exploration depend on the knowledge of pathogenesis and development of countermeasures for a variety health risks associated with space flight. Among the first line of health challenges are space flight induced anorexia, weight loss, and atrophic/degenerative musculoskeletal changes that are apparent, even during relatively short-term space missions [3,11].

Despite generally low energy expenditure and consumption of nutritionally balanced food, most astronauts in spaceflight missions experience a net deficit in energy intake that causes up to 5% net loss of body weight. Approximately 55% of this loss is attributed to total body adipose tissue, whereas 30% is attributed to water loss, and a further 15% reduction is due to bone and muscle tissue losses [8,22,25,3,33]. Even with intense in-flight training exercise programs, the space flight crew members experience an average of up to 3% of monthly declines in lower limb BMD and muscle volume and force capacity [3,11].

Multiple in-flight measurements and studies in ground based human (head-down bed-rest model) and animal (HLS) models of microgravity [8,25,27] identified microgravity and limb disuse as primary causes of inflight musculoskeletal deficiencies. In addition, the observation of human subjects [8] and studies in animals [10,19,30,33,36] exposed to sub-lethal doses of irradiation (IR) had identified anorexia as one of common components of acute radiation injury syndrome suggesting that it could also contribute to development of space flight anorexia and weight loss [8].

Overall however, mechanisms by which these deficiencies occur in the space environment are not completely understood. In particular it remains uncertain if effects of irradiation exposure and

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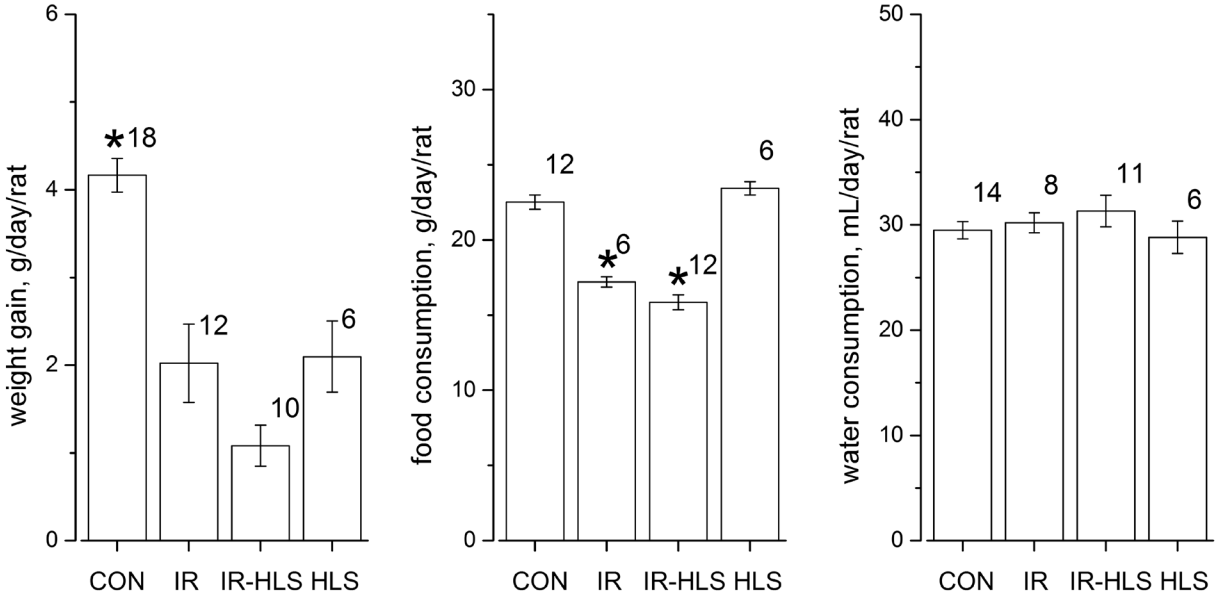

Figure 1. *Mean values of daily weight gain* ***(A)****, and food* ***(B)*** *and water* ***(C)*** *consumption during two weeks of experiment by control (CON) rats, or those that were either Hindlimb suspended (HLS), Irradiated (IR), or both (IR-HLS). Numbers in each panel indicate numbers of rats per group (*= p<0.05 from CON).*

those of the microgravity environment on feeding behavior, body weight, and muscle and bone strength are independent and additive or may exacerbate each other. Even to date, the most studied question regarding space-induced bone loss remains unanswered with some studies reporting trabecular bone loss after IR and HLS with amplification of this effect in irradiated and then suspended rodents [24,37], while others concluding that IR and HLS share a common cellular mechanism of bone resorption [21]. Differences in sequence of procedures (IR followed by HLS vs. HLS followed by IR), type and dose of IR (X-rays, protons, heavy ion particles; 0.5–4 Gy), duration of HLS (1–4 weeks), species (rat vs. mouse), studied bone regions, and outcome measures in these and other studies [2,21,24,37,38], all might have contributed to this uncertainty. Perhaps, the most important however, is that in all of these studies the effects of a single IR exposure preceding or following relatively prolonged period of HLS were compared. In none of these studies was animal weight gain and food consumption monitored daily or information on muscle loss/gain available.

Thus, the major focus of this work was to measure and compare weight gain, food and water intake, and indices of musculoskeletal system (soleus muscle weight and hindlimb bone parameters) in four groups of rats: control (CON), irradiated (IR, X-rays), hindlimb suspended (HLS) and suspended and irradiated (IR-HLS). As opposed to a single exposure protocols, we chose fractionated dose repeated X-ray exposure. The radiation was delivered in five sessions during the 2 week study period with HLS animals remaining suspended during exposures. This protocol mimics the clinical scenario of some cases of radiation therapy. Furthermore, with the 1.2 Gy/session dose (cumulative dose 6 Gy) chosen, this protocol may be expected to replicate the total whole body exposure that would occur during large solar particle events (lasting 8-24 h) in combination with galactic cosmic radiation background during space travel [35].

Materials and Methods

All animal protocols were approved by the Institutional Animal Care and Use committee and experiments were conducted in accord with the National Institute of Health Guide for the Care and Use of Laboratory Animals. Adult male Sprague-Dawley rats (200–300 g., Harlan Inc., Indianapolis, IN) were used in all experiments. Animals had free access to the tap water and food pellets (Harlan Teklad rodent diet #8640, Madison, WI; calories: 29%, 17% and 54% from protein, fat and carbohydrates, respectively). Body weights and water and food intake were measured at baseline and daily (between 9:00AM and 10:00AM) until euthanasia. After one week of acclimation to the animal care facilities, rats

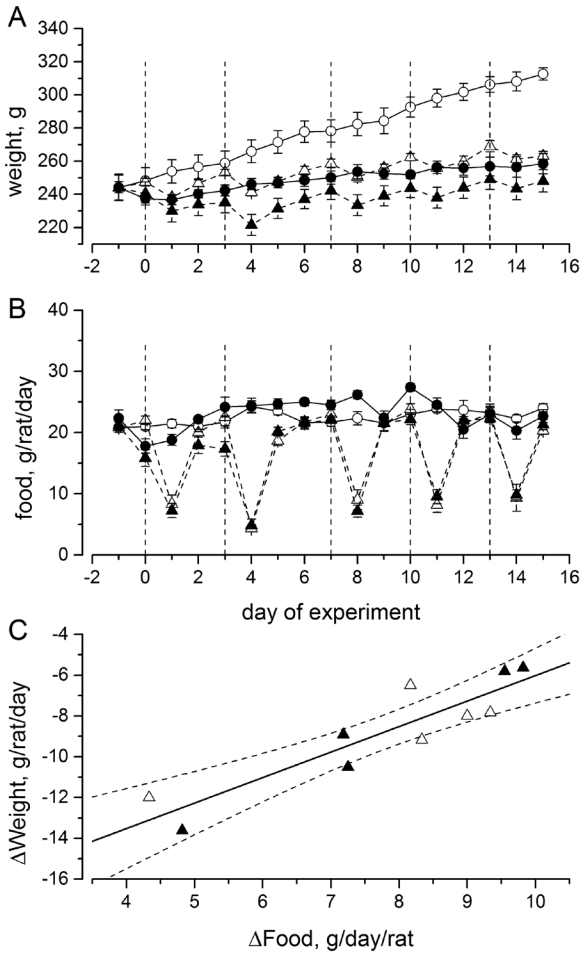

Figure 2. *Effects of HLS and IR on weight* ***(A)*** *and food consumption* ***(B)*** *and the relationships between post-irradiation food intake and weight changes (C). Open circles (Control), closed circles (HLS), open triangles (IR), and closed triangles (IR-HLS).* ***(A*** *and* ***B)*** *Vertical dashed lines mark days of irradiation sessions. The day of the first irradiation session is set to day zero. In HLS and IR-HLS groups, HLS was conducted on the day before the first irradiation session (day -1).* ***(C)*** *Solid and dashed straight line is regression line for the post-irradiation IR and IR-HLS (opened and solid triangles, respectively) group data, combined. Linear fit procedure suggests 1.2±0.2 g of the weight change per 1g of the food consumed (adjusted R squared is 0.804. Dashed lines are regression line 95%CI.*

were randomly assigned to individually-housed control (CON) irradiation (IR), irradiation and hindlimb suspension (IR-HLS), and hindlimb suspension (HLS) groups. A total of 61 animals, 3 to 18 per group were studied.

Hind limb suspension (HLS). Rats were briefly (for 2-3 minutes) anesthetized by isoflurane inhalation for placement of the tail harness as previously described [6,7] using a tail harness constructed by looping a strip of Skin-Trac orthopedic foam (Zimmer Inc., Charlotte, NC) around a pulley that can travel along a bar traversing the length of the cage with one important modification. The adhesive surfaces of the reminder of the foam strip were applied to the long axis of not the opposite sides but to dorsal and ventral surfaces of the tail, creating a "tail-sandwich", secured by enwrapping it with orthopedic stockinet and three half-inch wide pieces of glass zip-reinforced strapping tape at the base, middle, and few centimeters from the tip of the tail. After the animal had fully recovered from anesthesia, rats were suspended in individual plastic cages for two weeks at about 30 degrees head-down tilt. The angle of suspension was adjusted to ensure that when the animal was fully stretching its hind limbs, it was unable to touch the ground.

Radiation Exposure. In the main set of experiments, rats from IR and IR-HLS groups were exposed to whole-body X-ray irradiation in 5 sessions (radiation dose of 1.2 Gy per rat per session) given at 3-4 day intervals over 2 weeks (N=6–18 rats per group). To determine dose-response relationships between irradiation and food intake / weight gain, an additional set of experiments was conducted, in which control animals (N=4) were compared to IR rats (N=6) that were exposed to escalating doses of 0.2, 0.3, 0.4, 0.6 and 0.9 Gy of X-rays at 3-5 days intervals between sequential sessions. Finally, 3 more animals were exposed to 0.5 Gy X-rays to verify an important ED50 point in the "IR dose–weight loss" relationships obtained in the study above.

For each IR session, rats were transported to the radiation facility between 10:00AM and 11:00AM in their home/suspension cage. The radiation was delivered one rat at the time using 160 KVp X-rays generated by a Faxitron cabinet X-ray system (Lincolnshire, IL, USA) apparatus. A rat was placed into a special exposure chamber (25 cmx25 cm height x diameter glass vial; in which HLS rats remained suspended and non-suspended rats were allowed to move freely. An exposure to an X-ray dose of 1.2 Gy to the base of the chamber at feet level was given at a dose rate of 0.6 Gy /min, while the sham radiation procedure was performed over the same amount of time in the same machine without any X-ray exposure. After exposure, the animal was immediately returned to their home cage. The dosimetry for this system was as described previously and is similar to other studies that have examined the effects of space and therapy-related radiation exposures [35,37].

Bone mineral density (BMD) measurements by dual energy X-ray absorptiometry (DEXA). Longitudinal bone densitometry was performed using the Piximus 2 Bone Densitometer (Lunar Corp., Madison, WI); as described previously [9]. Scans were performed on the right hindlimb under anesthesia on day 14 immediately prior to euthanasia. Regions of interest (ROI) were drawn on each hindlimb scan that divided the tibia or

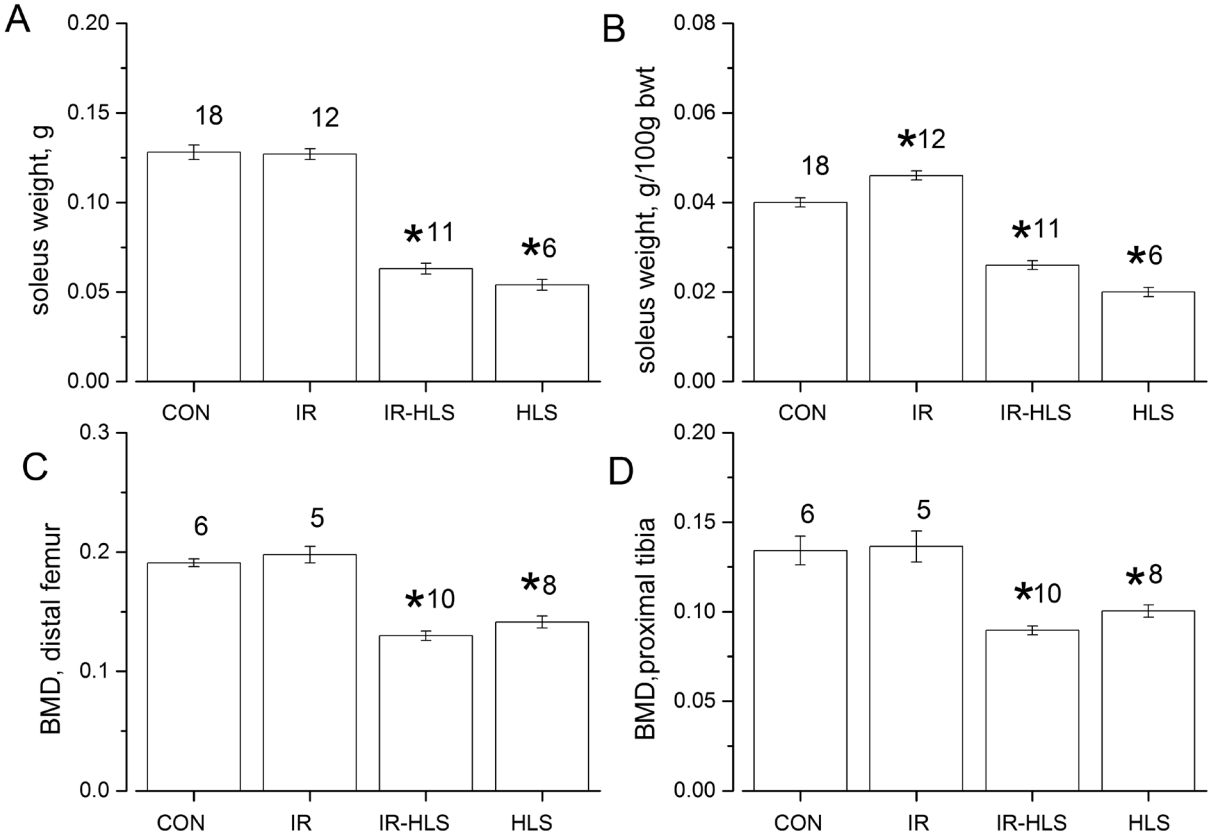

Figure 3. *Effects of IR and HLS on soleus muscle wet weight* ***(A)*** *and muscle/body weight ratio* ***(B)****, as well as effects on the BMD of the distal femur* ***(C)*** *and proximal tibia* ***(D)****.Values above the bars indicate number of rats per group. *= p<0.05 different from CON.*

femur into the proximal and distal one-thirds (comprised of both cortical and trabecular bone). Bone mineral density (BMD) was determined for each ROI, to enable comparison of changes in trabecular + cortical bone, as previously described [9]. The precision and accuracy of the Piximus instrument have been determined by repeated measurements of 5 animals, 5 times each. In-house precision analyses have been previously determined for adult rat femoral BMD to be 0.1% CV.

Statistical analysis. Statistical analysis was conducted using Statistica Software (StatSoft, Tulsa, OK). The data were analyzed for normality of distribution (Shapiro-Wilk test), and then analyzed using One- or Two-Way ANOVA, followed by Tukey's post-hoc comparison tests. Linear regression and non-linear best-fit analysis was conducted using Levenberg–Marguardt minimization algorithm (Origin 9.0; OriginLab, Northampton, MA), with all parameters set free to vary. Effects were considered as statistically significant at $p<0.05$. Data in figures are expressed as mean ± SE.

Results

Weight gain and food and water intake. Changes in weight/weight gain and food and water intake are indirect indicators of stress experienced by experimental animals. Mean rate of the weight gain by control rats during 2 weeks of experiment approximated 4 g/day/rat. Both IR and HLS alone independently reduced weight gain by ~50%, which was further reduced in IR-HLS group of rats (although not reaching significance) **(Figure 1A)**.

While mean water consumption did not differ between control and experimental groups of rats **(Figure 1C)**, irradiation statistically significantly suppressed average food intake in both suspended (IR-HLS) and non-suspended (IR) rats **(Figure 1B)**.

Daily analysis of body weight changes and food intake in studied groups of rats **(Figure 2A** and **B)** shows that weight of irradiated animals drops acutely immediately following the acute and transient drop in food intake that occurs following each irradiation session (vertical dashed lines in **Figure 2A** and **B**). Weight recovery to a pre-irradiation level occurred on the second-third post-irradiation day. Furthermore, linear regression analysis of relationships between food consumption and weight gain on individual post-exposure days suggested existence of direct proportionality between these

parameters in irradiated rats (R^2=0.804, **Figure 2C**). Finally, it is important to note that while there was apparently no accumulating effect of repeated IR sessions on reduction in food intake, as it recovered to baseline level within two days after each exposure, the effect of IR on rat weight gain was completely inhibitory. Although IR rats were able to recover their weight to a control level after the first IR exposure, the weight of IR rats never increased beyond the baseline in contrast with the continual weight gain of CON rats throughout the 2 week experiment **(Figure 2A)**.

Muscle atrophy and bone loss. Unlike the deleterious effect of IR on food intake and body weight gain, no statistically significant degenerative effects of irradiation alone on soleus muscle weight or long bone BMD were detected in our experiments. Both soleus muscle weight **(Figure 3A)** as well as distal femur and proximal tibia BMD showed the expected reductions in response to HLS **(Figure 3C** and **D)**, although no differences in midshaft cortical BMD were observed (data not shown). However, muscle weight and BMD did not change after IR alone, and IR did not add to the HLS-induced bone loss in combination with simulated microgravity and IR. Surprisingly, however, when the data were normalized to the rat body weight to soleus muscle weight, IR appeared to increase muscle weight by 15% - 30% (IR and IR-HLS groups, respectively). The difference in normalized soleus weight of control and IR animals was statistically significant **(Figure 3B)**. There is however uncertainty as to what degree this "stimulating" muscle mass effect of IR is confounded due to incomplete recovery of the rat body weight (the denominator in normalized mass calculations) after the last irradiation session, and prior to the endpoint sample collection in these experiments.

X-ray irradiation – food intake/weight gain dose-response relationships. Additional experiments were conducted to determine the threshold dose of X-ray irradiation required to alter rat weight and food consumption. The results of these experiments combined with the results of studies of effects of 1.2 Gy exposures are shown in **Figure 4**. The X-ray dose-food intake relationships could be best fit by linear function suggesting no minimal safe dose of the irradiation **(Figure 4A)**. In contrast, 0.3 Gy dose was the minimal dose required to observe beginning of the weight-loss effect and this effect leveled off after 0.7 Gy exposure dose **(Figure 4B)**.

Discussion

The question of whether exposure to radiation and microgravity during space flight act independently or in concert to produce such complications as anorexia, body weight, and muscle and bone loss during a space flight remains unresolved. As a first step of addressing this question, we evaluated effects of relatively high dose of X-ray irradiation (five exposures of 1.2 Gy each) on food and water consumption, body weight, BMD, and soleus muscle weight of normal control and HLS rats (ground-based model of microgravity). These types of radiation doses are likely on the high end of what would occur during a particularly active space flight with regard to solar particle events. However, our studies suggest that at least with regard to the effect on food consumption there is no safe dose of the exposure and that noticeable effect on body weight could also be observed at space flight relevant exposures (0.3-0.5 Gy). In light of this finding, studies using other space flight relevant radiation exposure regimens (i.e., mixed particle, longer times, lower exposure rates) are warranted to further delineate the potential association of space irradiation and microgravity on weight gain, food intake, and musculoskeletal atrophy.

In the part of experimental microgravity and muscle disuse, we confirmed previous observations of deteriorating effects of HLS on rat body weight, postural muscles (soleus), and rat hind limb bone parameters [5,2,37]. We have also observed only a small but transient net weight loss of HLS animals with no effects of HLS on mean daily water or food intake. These observations are also consistent with previous data, showing that when detected, such changes are small in magnitude and constrained to a few days that are required for the animal to adapt to a new environment of HLS [5,8,27].

In general, our study is also in agreement with previous studies of the effects of irradiation on weight and food consumption by rats, by confirming that a relatively low dose of whole body IR (0.3–0.7 Gy; **Figure 4**)) results in acute reduction in both food intake and weight loss [10,23,30,36] and references within)). The major difference in those reports and our study is the timing of onset and duration of post-irradiation changes in studied parameters. To a large extent this variability is likely due to relative differences in type of irradiation source and dose

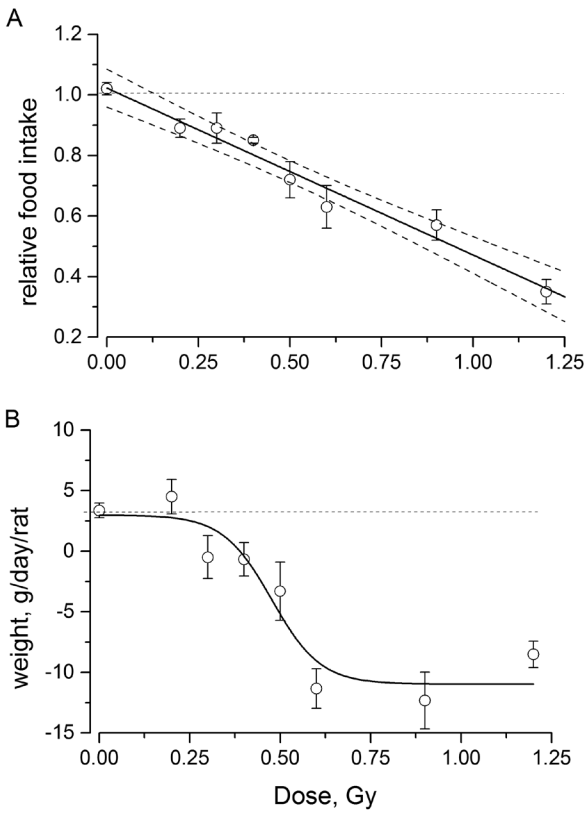

Figure 4. *X-ray dose – food intake and body weight change relationships.* ***(A)*** *post-exposure food intake (relative to that during pre-exposure day); Linear fit (solid line ± 95%CI) parameters: intercept=1.02±0.03, slope -0.33±0.03 (33% food consumption decrease per 1 Gy); Adj R^2=0.958.* ***(B)*** *weight change (difference between pre- and post-exposure days); Sigmoidal fit (solid curve) parameters: maximum weight drop 11±2 g, half-effective X-ray dose = 0.48±0.05 Gy; Adj R^2=0.842. In both graphs, the value plotted at zero of the X-axis represents the mean of results obtained in all sham-exposed rats in this study (n=16). Data for 1.2 Gy exposure dose are mean values for all five sequential per rat exposures (n=12) and data for 0.5 Gy exposure are mean values for three animals.*

used. For example, 0.75 Gy of γ-irradiation (Co^{60} source) was reported to result in both weight gain and food intake decreases, limited strictly to the period of irradiation [10]. The 0.9 Gy [36] and 0.7– 1.2 Gy X-ray doses (first IR session in our study) resulted in an acute drop in food intake lasting for 24 hours post-exposure. However, 2.9 and 4.8 Gy X-ray and 4.5 Gy γ-ray and proton irradiation were reported to produce acute, but lasting effect for at least 4 days, post-exposure decreases in food intake [30,36]. Yet arguing against a simple dose-response relationship, the very recent publication by Lee et al. [23] claims that with a single 15 Gy dose, whole abdominal irradiation diminishes rat body weight by the 7th post-irradiation day, whereas food intake decreased in the same animals starting on day 1 and continuing through the 30th post-irradiation day [23]. Our group has published similar findings in a partial body irradiation study which could have relevance to certain exposures encountered on space walks or during intense solar particle events where one portion of the body is shielded by the spacesuit and equipment much more than other parts of the body [15,16].

We did not detect any changes in mean water intake by irradiated normal (control) or HLS rats. This issue is certainly controversial and requires further studies. A decrease in water intake during exposure to 0.75 Gy γ-ray irradiation and normal or increased water intake during 24 hr period after the first and 8th IR exposures, respectively was reported in one study [10]. In another study, the variable in direction (increase, decrease or no change) and duration (one to six days post-exposure) effect on water intake following a single exposure to X-rays was observed, with the number of rats demonstrating post-irradiation polydipsia increasing with the dose of radiation [30]. Whatever the actual effect of IR on water intake is, our data suggests that it is independent of irradiation-induced anorexia.

The novel and important findings of our work is clear dissociation of pathogenic consequences of HLS and IR. Although both HLS and IR exposure resulted in about 50% growth retardation effects, those appeared to be governed by different and independent mechanisms, with combined IR-HLS resulting in the weight gain reduction up to 25% of that in control rats and with post-IR weight gain/loss in IR animals being directly proportional to the amount of food intake **(Figure 2)**. Mechanisms of IR- and HLS-induced weight changes are not fully understood, although they are very likely to be different. The former is generally thought to result from the acute effect of IR on satiety signaling, such as decrease in gastric ghrelin release [23] or in modulation of cholecystokinin release and hypothalamic concentration [19]. The HLS-associated weight gain retardation, however, occurred with no changes in food intake (**Figure 1** in this work and [5]), which agrees with the suggestion that it is governed by the deficiency in protein utilization rather than energy intake [18,32]. Furthermore, while HLS affects both lean and fat body mass, it was demonstrated that at a relatively high dose of 15 Gy of IR (abdominal IR) loss of the fat mass was about 4 times greater than that of lean mass [15].

Although a decreases in food intake and weight loss following exposures to 1.2 Gy correlated each other **(Figure 2C)** and the "X-ray dose - food intake" relationships were linear **(Figure 4A)**, the "X-ray dose – weight loss relationships" appear saturated at an IR dose exceeding 0.9 Gy **(Figure 4B)**. This suggests existence of some reversibly depleted body compartment by IR (perhaps fat); see above). Alternatively, compensatory slowing of the rate of metabolism and/or animal activity could have been a factor. The latter scenario appears to be more likely as fat turnover rate is too slow (0.5-1 g/100 g rat body weight; [26]) to explain observed fast post-IR decrease by us and subsequent recovery of animal weight. Indeed, effects of a single, 7 Gy dose of γ- irradiation on mouse fat pad weight and adipocyte proliferation have been reported to develop within 4 days and persist for 12 post-IR days [29]. For the comparison, suppression of the rat open field exploratory behavior by 10 Gy γ-irradiation appear to be transient (complete recovery within 3 days, [20]which parallels the transient effect of X-ray irradiation on body weight in our study.

Another remarkable difference between HLS and IR data is that despite the profound effect of IR on both food intake and weight gain; it did not affect either net soleus mass or BMD. With regard to the soleus weight, the lack of direct effect of IR is not surprising as skeletal muscles are generally resistant to IR [17].

The effects of IR and HLS on bone are quite intriguing. DEXA analysis in the current study **(Figure 3)** detected loss of trabecular bone in HLS but not in IR animals. In agreement with this observation, three-point bone bending analysis also demonstrated decreases in bone peak load and stiffness in HLS rats, but no further deterioration in these parameters by additional IR treatment [40].

Our data are distinct from previous reports of bone loss in response to IR. As much as 16%-18% of cancellous bone loss (tibia) was observed by microCT analysis in experiments in mice 3-10 days after a single dose of 0.1-0.5 Gy heavy particle, ^{56}Fe [38], or 1-2 Gy gamma, ^{137}Cs [21]. Furthermore, recently, a single dose of X-rays (4 Gy) was reported to produce 57% loss in volumetric BMD male Wistar rat proximal tibia trabecular region at 4 weeks post-exposure [37]. Furthermore, caloric restriction (1–2.5 months) was reported to decrease rat femur BMD by 10% to 20% in adult rats [14,34]. Thus, in addition to a direct effect of IR its anorexic effect could also be expected to result in a bone loss. Further studies are needed to determine if this discrepancy may be attributed to the between-studies variations in the type of IR, dose, duration of experiment, or protocol of exposure, Additionally, DEXA analysis is less sensitive than micro-CT analysis used in the studies above. It is, however, important to note that DEXA in our study was sensitive enough to detect bone changes in HLS animals, firmly suggesting that under mixed microgravity/IR conditions, bone effects of microgravity outweigh the effects induced by IR.

In conclusion, with regard to pathophysiological complications associated with exposure to radiation our data are relevant to complications experienced by whole body cancer radiotherapy patients, treated with 1.0 Gy and higher doses of irradiation [4,12]. Also, our results suggest also that even with acute X-ray exposure, a decrease in food intake is introduced with very low doses. Moreover, both IR and HLS independently induce major physiological changes in the musculoskeletal system, including decreases in muscle mass, trabecular, and cortical BMD. Thus, exposure to space radiation is likely to contribute to varying degrees to the degenerative processes described here, depending on the solar flare activity and length/distance of overall mission. On the other hand, space radiation may be a constant and critical factor of maintained space flight anorexia and negative energy balance. Further studies utilizing a variety of IR regimens and proton and HZE radiation sources are warranted to address this issue.

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