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Mimicking a space mission to Mars using hindlimb unloading and partial weight bearing in rats

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Journal of Visualized Experiments

To the Editors:

Please consider our original research article entitled, “Mimicking a space mission to Mars using hindlimb suspension and partial weight bearing in rats”, for consideration for publication in the Journal of Visualized Experiments as per your invitation. This is the first study that aimed at developing a ground-based analogue to mimic a space mission to Mars using both hindlimb suspension and partial weight bearing. This article is an original publication and has not been submitted elsewhere.

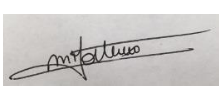
This study is based on our previous findings published earlier this year in the Journal of Applied Physiology regarding a novel quadrupedal partial gravity analogue in rats, allowing longitudinal investigations. This novel analogue, combined to a modified hindlimb suspension protocol, now allows us to mimic complete space missions including different environments. Indeed, this protocol can be adapted to investigate the effects of microgravity, partial gravity, partial reloading, and total reloading in an infinite number of settings. Moreover, it highlights for the first time the effects of partial gravity on animals previously exposed to microgravity (mechanical unloading).

This innovative analogue will provide a suitable model for ground-based studies investigating the effects of both short and long duration spaceflights and/or space missions, and will bring crucial insights regarding the physiological changes occurring in other planets such as Mars.

Thank you for your consideration of this article for publication in the Journal of Visualized Experiments.

Sincerely yours,

Dr. Marie Mortreux



TITLE:

Mimicking a Space Mission to Mars Using Hindlimb Unloading and Partial Weight Bearing in Rats

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KEYWORDS:
space flight, partial gravity, Mars, hind limb unloading, partial weight-bearing, muscle

SUMMARY:
By using an innovative ground-based analogue model, we are able to simulate a space mission including a trip to (0 g) and a stay on Mars (0.38 g) in rats. This model allows for a longitudinal assessment of the physiological changes occurring during the two hypo-gravitational stages of the mission.

ABSTRACT:
Rodent ground-based models are widely used to understand the physiological consequences of space flight on the physiological system and have been routinely employed since 1979 and the development of hind limb unloading (HLU). However, the next steps in space exploration now include to travel to Mars where the gravity is 38% of Earth’s gravity. Since no human being has experienced this level of partial gravity, a sustainable ground-based model is necessary to investigate how the body, already impaired by the time spent in microgravity, would react to this partial load. Here, we used our innovative partial weight-bearing (PWB) model to mimic a short mission and stay on Mars to assess the physiological impairments in the hind limb muscles induced by two different levels of reduced gravity applied in sequential fashion. This could provide a safe, ground-based model to study the musculoskeletal adaptations to gravitational change and to establish effective countermeasures to preserve astronauts’ health and function.

INTRODUCTION:

Extraterrestrial targets, including the Moon and Mars, represent the future of human space exploration, but both have considerably weaker gravity than Earth. While the consequences of weightlessness on the musculoskeletal system have been extensively studied in astronauts[1–5] and in rodents[6–9], the latter thanks to the well-established hindlimb unloading (HLU) model[10], very little is known about the effects of partial gravity. Martian gravity is 38% of Earth's and this planet has become the focus of long-term exploration[11]; hence, it is crucial to understand the muscular alterations that may occur in this setting. To do so, we developed a partial weight bearing (PWB) system in rats[12], based on previous work done in mice[6,13], which was validated using both muscle and bone outcomes. However, the exploration of Mars will be preceded by a prolonged period of microgravity, which was not addressed in our previously described model[12]. Therefore, in this study, we altered our model to mimic a trip to Mars, comprised of a first phase of total hindlimb unloading and immediately followed by a second phase of partial weight bearing at 40% of normal loading.

Unlike most HLU models, we chose to use a pelvic harness (based on the one described by Chowdhury et al.[9]) rather than a tail suspension to improve animals' comfort and to be able to move seamlessly and effortlessly from HLU to PWB in a matter of minutes. In conjunction, we used the cages and suspension devices that we previously developed and described extensively[12]. In addition to providing reliable/consistent data, we also previously demonstrated that the fixed attachment point of the suspension system at the center of the rod did not prevent the animals from moving, grooming, feeding, or drinking. In this article, we will describe how to unload the animals' hind limbs (both totally and partially), verify their achieved gravity levels, as well as how to functionally assess the resulting muscular alterations using grip force and wet muscle mass. This model would be extremely useful for researchers seeking to investigate the consequences of partial gravity (either artificial or extra-terrestrial) on an already compromised musculoskeletal system, thus allowing them to investigate how organisms adapt to partial reloading, and for the development of countermeasures that could be developed to maintain health during and after human spaceflight.

PROTOCOL:

All methods described here were approved by the Institutional Animal Care and Use Committee (IACUC) of Beth Israel Deaconess Medical Center under protocol number 067-2016.

NOTE: Male Wistar rats aged of 14 weeks at baseline (day 0) are used. Rats are housed individually in custom cages 24 h prior baseline to allow for acclimation.

1. Hindlimb unloading

NOTE: The pelvic harness can be put on either anesthetized or awake animals. Here, the description of the protocol is given on anesthetized animals. Wear proper personal protective equipment (PPE) to handle animals.

1.1. Place the rat in an anesthesia box with 3.5% isoflurane and an oxygen flow of 2 L/min.

NOTE: Proper anesthetization is confirmed when a firm pinch of the rear paw does not elicit a reaction.

1.2. Once the animal is fully anesthetized, place the rat on the bench with anesthetic gas coming from a nosecone with 2% isoflurane and an oxygen flow of 1.5 L/min.

1.3. Place the rat in a prone position and put the pelvic harness on in a rostro-caudal movement.

1.4. Gently bend the pelvic harness to provide a snug fit while being careful not squeeze the hindlimbs to prevent abrasions and discomfort.

1.5. Attach the stainless steel chain with the swivel clasp to the top of the pelvic harness, where a hook is attached at the base of the tail.

1.6. Remove the rat from anesthesia and place the animal in a custom cage with the chain extended at its maximum.

1.7. Once the rat is fully awake and mobile, shorten the chain using the top swivel clasp until the hind limbs can no longer reach the floor.

1.8. Observe the animal for a few minutes to assess its comfort and make sure that at all times, both hind limbs remain completely unloaded.

2. Partial weight bearing

NOTE: This step can be realized in both awake and anesthetized animals.

2.1. Convert the HLU suspension device into a PWB suspension by adding the triangle-shaped part composed of stainless steel chains and a back rod.

2.2. Anesthetize the animal following the same procedures as detailed for the HLU (steps 1.1 and 1.2).

2.3. Place a tether jacket of the appropriate size on the forelimbs of the rat (M for rats of 400 g or lower, L for rats weighing above 400 g) and close it using the back bra extender.

2.4. Attach one clasp of the triangle-shaped part to the hook located on the back bra extender and the opposite clasp on the hook located on the pelvic harness at the base of the tail.

2.5. Allow the animal to recover from anesthesia in the cage. Once awake, verify that the suspension is equal on both the forelimbs and the hindlimbs by shortening the chain and modifying the location of the bottom swivel clasp if needed.

NOTE: This step can also be realized using a force plate to confirm the equal loading on all limbs.

2.6. Place the rat on top of the scale to record the "loaded" body weight, i.e., the weight of the animal and the entire apparatus, without shortening the chain.

2.7. Shorten the chain until the scale displays 40% of the "loaded" body weight and record the achieved gravity level (expressed as the ratio between unloaded weight and loaded weight).

2.8. Observe the animal to make sure that the unloaded weight is stable and that the rat is equally loaded on all limbs.

2.9. Remove the entire apparatus from the scale using the rod and place the rat back in its cage.

3. Assessment of hindlimb grip force

3.1. Hold the rat with a traditional restraint by placing one hand underneath the forelimbs. Gently hold the tail with the second hand.

3.2. Approach the grip bar with the rear paws and make sure that both paws are fully resting on the bar.

NOTE: If the rat does not fully grip the bar or does not display any evidence of voluntary gripping, slightly release the restraint. If this is unsuccessful, return the rat to its cage and retry after a few minutes.

3.3. Gently pull the rat straight back until it releases its grip. Record the maximal force displayed on the transducer.

3.4. Wait approximately 30 s between measurements and repeat the test 3 times.

3.5. Calculate the average of the three measurements for scoring, to account for fatigue.

4. Recording of muscle wet mass

4.1. Place the rat in a CO_2 euthanasia chamber. After waiting the appropriate time according to IACUC guidelines, confirm euthanasia by a visual observation of a lack of breathing.

4.2. Place the rat on the dissection table in a prone position and remove the fur and skin by incising near the ankle using small dissection scissors. Use hands to pull the skin layer off.

4.3. Using small dissection scissors, gently break the muscle fascia and isolate the calcaneus tendon.

NOTE: The calcaneus tendon is the attachment point of both the soleus and the gastrocnemius muscles.

4.4. While holding the calcaneus tendon with a small pair of tweezers, use the dissection scissors to isolate the gastrocnemius and soleus muscles from the biceps femoris, located above.

4.5. Once isolated, cut the attachment point of the gastrocnemius and soleus muscles in the popliteal area.

4.6. Gently pull the soleus away from the gastrocnemius and detach them by cutting the calcaneus tendon.

4.7. Place the rat in a supine position. Carefully remove the fascia and peel the tibialis anterior from the ankle in an upward movement.

4.8. Cut the tibialis anterior at its superior attachment point.

4.9. Record the exact wet mass of each excised muscle using a tared precision scale and a weighing boat.

REPRESENTATIVE RESULTS:

Taking advantage of the new cages that we previously designed and described in detail[12], we used a stainless steel chain-based suspension device that is suitable for both hindlimb unloading (HLU, **Figure 1**) and partial weight-bearing (PWB, **Figure 2**). The critical advantage of our design is the ability to go from one type of unloading to the other in a matter of minutes while maintaining an identical environment for the animals. We used a custom-made pelvic harness (**Figure 2A**) that is attached to a single custom-made stainless steel chain with a swivel clasp on each side for HLU. In order to modify this suspension device and achieve PWB, the addition of one triangle-shaped piece of stainless steel chain that incorporated an inflexible back rod, designed to sit just above the spine (**Figure 3**) is the only requirement. These steps can be performed in awake or anesthetized animals.

With the versatile environment provided in this experiment, we could successfully unload the hindlimb of all of our animals for 7 days without any complications and quickly expose them to a partial gravity at 40% of their normal loading (PWB40, average achieved gravity level of 0.4076 g ± 0.0036 g). During the first week of total HLU, animals displayed a significant body weight loss (**Figure 4A**: -7.19% ± 0.87%, n = 9, $p < 0.001$), which has been witnessed in other models[14], and does not differ significantly from what we observed in rats exposed at PWB40 for the same duration (-5.53% ± 1.44%, n = 10, $p = 0.37$). However, animals continued losing weight over time while subsequently being exposed to PWB40 (-9.06% ± 1.35% from baseline, $p < 0.0001$).

Hindlimb grip force is a standard measurement of muscle function that can be used longitudinally (**Figure 4B**). We noticed that one week of total unloading led to an average decrease in grip force of 50.16% ± 4.10% compared to baseline ($p < 0.0001$). After one subsequent week of partial weight bearing at 40% of normal loading, we did not notice any further change regarding grip force (-44.29% ± 4.67% compared to baseline, $p < 0.0001$). At all time-points, the percent change in rear paw grip force was significantly different from the age-matched controls ($p < 0.0001$ for both day 7 and day 14, n = 11). Additionally, we observed that after completion of the study, animals that underwent total unloading followed by partial weight bearing (HLU-PWB40) displayed a significantly greater grip force loss compared to the PWB40 group ($p = 0.03$).

Muscle wet mass was recorded at the end of the experiment and compared to data obtained after either two weeks of normal loading or two weeks of PWB40 (**Figure 4C**) and data previously published by our group[12]. We found that the PWB40 and HLU-PWB40 groups have significantly lower wet mass of soleus (S), gastrocnemius (G), and tibialis anterior (TA) muscles than age-matched controls (PWB100). Indeed, we recorded an average soleus mass of 0.1681 g ± 0.007 g for our animals which was significantly lower than the rats exposed to PWB100 for 2 weeks in our previous experiments (-24.60% ± 3.18%, $p < 0.0001$). For the gastrocnemius, we recorded an average wet mass of 2.192 g ± 0.096 g (-10.55% ± 3.93%, $p = 0.038$ vs PWB100) and a wet mass of 0.759 g ± 0.029 g for the tibialis anterior (-14.40% ± 3.27%, $p = 0.009$ vs PWB100). While our data set highlighted that the animals exposed to a Mars-mission analogue (HLU-PWB100) had a decreased wet mass of the soleus and gastrocnemius muscles compared to the animals exposed to PWB40 for 2 continuous weeks (-8.75% ± 3.84% and -5.85% ± 4.14%, respectively), we did not observe a significant difference between these two groups.

FIGURE LEGENDS:

Figure 1: Description of the suspension devices and how to convert from HLU to PWB. (**A**) Based on our previous design, we used an aluminum rod sitting on top of the cage to hold a steady suspension device composed of a key ring secured at the center of the rod (arrow 1), a stainless steel chain (arrow 2), and two swivel clasps (arrows 3). (**B**) To convert the suspension device to achieve PWB, a triangle-shape structure is attached using the bottom swivel clasp. This piece is composed of stainless steel chains and a polyvinyl chloride (PVC) back rod that sits above the rat's spine (arrow 1). On each side of the back rod is located a clasp to attach to the harness and the jacket, respectively (arrow 2).

Figure 2: Hind limb unloading using a pelvic harness. (**A**) Front and side view drawings of the harness structure used to support the hind limbs of the animals. (**B**) The pelvic harness was positioned as described to fit snuggly around the hind limbs of the rat. The stainless steel link is positioned over the base of the tail and attached to the swivel clasp. The exact location and shape of the harness can vary between animals but rats should be comfortable and it is necessary that their hind limbs never touch the ground.

Figure 3: Partial weight bearing. The partial unloading requires the addition of a jacket to the animal in order to support the front limbs. The jacket is then closed with a back bra extender and a hook is attached to the extender, lying between the scapulae. Both the jacket and the pelvic harness are attached to clasps located on each end of the back rod.

Figure 4: Examples of longitudinal follow-up in animals exposed to different unloading levels. (**A**) Body weight (BW) change. Animals were weighed weekly without the harness or jackets and body weight was recorded. PWB100 = Partial weight bearing at normal loading; PWB40 = Partial

weight bearing at 40% of normal loading; HLU-PWB40 = One week of hindlimb unloading followed by one week of PWB40. The results of Tukey's post hoc test following a 2-way stacked ANOVA are presented as *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, and ****: $p < 0.0001$ vs PWB100. (**B**) Change in rear paw grip force. Weekly, rear paw grip force was measured and results were expressed in percent change from baseline for each animal. The results of Tukey's post hoc test following a 2-way stacked ANOVA are presented as ***: $p < 0.001$ and ****: $p < 0.0001$ vs PWB100, α: $p < 0.05$ vs PWB40. (**C**) Muscle wet mass after 14 days. Muscle wet mass was recorded on a precision scale immediately after sacrifice at 14 days. Results are presented as percent of the wet mass obtained in the age-matched control group (PWB100). S= Soleus; G= Gastrocnemius; TA = Tibialis anterior. The results of Tukey's post hoc test following a one-way ANOVA are presented as *: $p < 0.05$, **: $p < 0.01$, and ****: $p < 0.0001$ vs PWB100.

DISCUSSION:

This model presents the first ground-based analogue developed to investigate successive mechanical unloading levels and aims to mimic a trip to and stay on Mars.

Many steps of this protocol are critical to ensure its success and need to be closely examined. First, it is crucial to monitor the wellbeing of the animals and ensure that they are maintaining a normal behavior (i.e., performing tasks such as eating, resting, and exploring), particularly during the PWB state where they maintain a relatively normal physiological posture. Second, despite the level of PWB being extremely steady over time and requiring minimal human intervention[12,14], it is essential to record the achieved partial gravity to minimize variations amongst animals. Additionally, when animals are transitioned from total mechanical unloading to partial gravity (PWB40), they already display substantial muscle atrophy and loss of function[6,9,15], which may cause transient difficulties in resuming quadrupedal weight-bearing behavior and lead to a momentary awkward gait.

Due to the variable environment, several problems can arise and should be closely recorded and addressed. For example, a fluid shift occurs during the HLU period due to the slanted position of the animal, while it is not present during PWB[16]. In some cases, fluid redistribution can cause subtle edema most noticeable in the face or the rear paws, and usually disappears in the hours following reloading. We advise investigators to score the severity of the edema and assess it daily. If severe edema persists for greater than 48 h, animals should be excluded from the experiment.

While the use of a pelvic harness provides comfort to the animals and increased convenience to the investigator, some animals may, occasionally, either totally or partially escape from their harness during HLU or PWB. We followed the exclusion protocol based on the previous work in mice[6] in which any animal that escapes three times is removed from the study. As a side note, escapes are extremely rare; in our work less than 1% of animals had to be excluded over a 1-year period (1 animal out of 148 animals studied). The daily titration of the PWB level is a crucial moment where the experimenter can ensure the snug fit of both the jacket and the pelvic harness, therefore minimizing the risk of escape. While assessing animals' weight and wellbeing daily, special consideration should be put in the maintenance of the pelvic harness. While site-specific hair-loss is the most common consequence, abrasion can appear if the pelvic harness is damaged (i.e., chewed). We advise researchers to check daily the harness condition and replace components when damaged or the entire harness when needed to prevent the appearance of skin abrasion.

The animals' claws can also occasionally become entrapped in the hook-and-loop fastener or cloth, therefore compromising their balance. A simple and efficient way to prevent this from happening is to gently trim the claws under anesthesia before placing the jacket. This step can be repeated when needed during the course of study.

Special attention needs to be paid during the transition from HLU to PWB. While we observed that all animals are able to walk with little difficulty immediately after being placed in PWB, the amount of time required to put the same amount of weight on both the front and the hind limbs varied among rats. If a rat does not demonstrate relatively normal gait using all limbs in 24 h, we recommend it should be excluded from the study.

This novel model designed to mimic sequential gravitational environments is reliable and sustainable over time. However, some limitations exist and are yet to be addressed. First, this combination of models is only designed to assess the alterations occurring in the hind limbs of the animals as the HLU model only creates artificial microgravity on the rear limbs. Therefore, this ground-based sequential HLU-PWB analogue is not suitable to investigate fore limbs alterations. Secondly, over the 14-day period, our animals displayed a continuous but non-life-threatening loss of whole-body mass highlighting the complex readjustment of the rats to partial unloading (**Figure 4A**). In our previous PWB rat model study, the animals exposed at PWB40 and PWB20 for two weeks presented a significant loss over only the first 7 days and regained weight subsequently[12]. This was likely due to the fact that the rats were able to adjust to the quadrupedal unloading after an initial period of adaptation. However, in this study, the rats never fully adapted to the two different unloading/partial-reloading periods of one week each, likely explaining the sustained weight loss. It would be important to further extend these periods of full and partial-unloading to confirm that animals can fully adapt and settle into each environment. Stress levels have not been evaluated in this model yet and could easily be monitored in the future using regular blood sampling using the tail that remains entirely accessible.

Our longitudinal assessments of muscle function and muscle mass showed that one week of hindlimb unloading caused a tremendous decrease in rear paw grip force (**Figure 4B**) with one of our rats exhibiting a 70% reduction in grip force. Unsurprisingly, after 14 days, animals displayed a significantly lower grip force than animals that had been exposed to 14 days of PWB40 in our previous study[12] Whereas the average wet mass of the hindlimb muscles did not differ significantly between the PWB40 and HLU-PWB40 groups, we were able to establish a strong linear correlation among our 3 groups (PWB100, PWB40, and HLU+PWB40) regarding the average soleus mass ($R^2 = 0.92$, $p < 0.0001$).

These results confirm that partial loading following a total mechanical unloading compromises muscle health more than what would be observed during a continuous but stable period of partial unloading. Until now, this gap in knowledge has not been investigated. Further assessment of this phenomenon should be pursued in order to develop effective countermeasures preventing muscle deconditioning in the context of a mission to the Moon or Mars. The strength of our model also resides in its versatility as it allows for a variety of different experiments with several degrees of unloading and for varying lengths of time.

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DISCLOSURES:

The authors have nothing to disclose.

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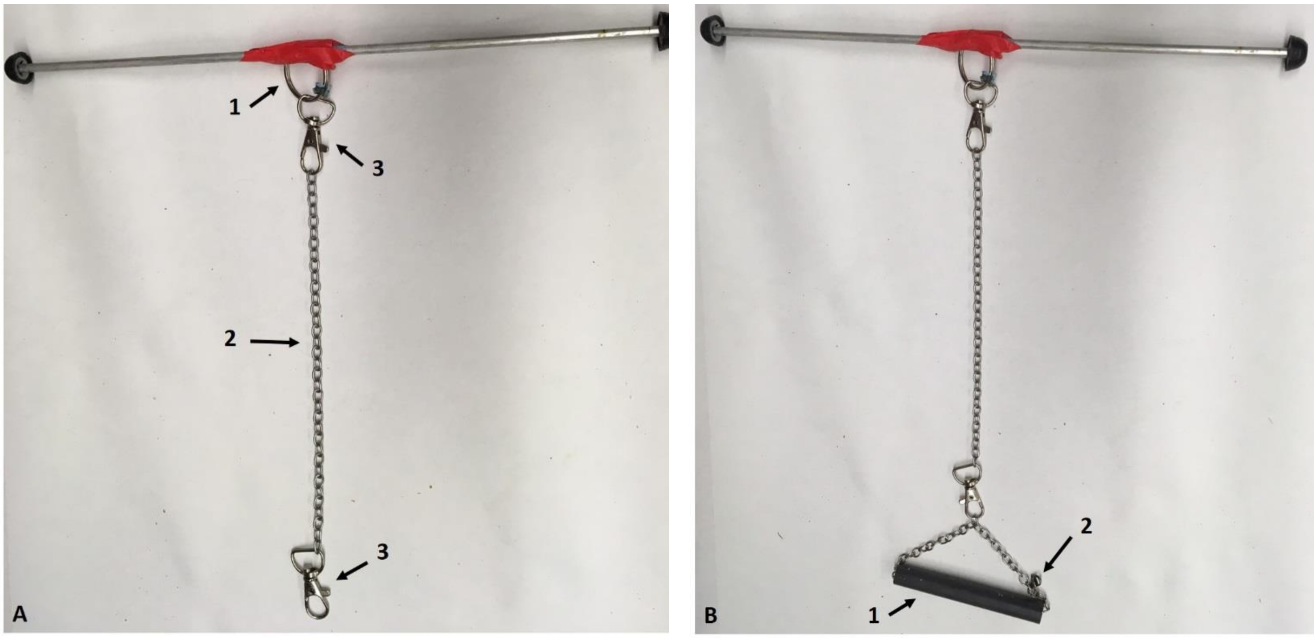
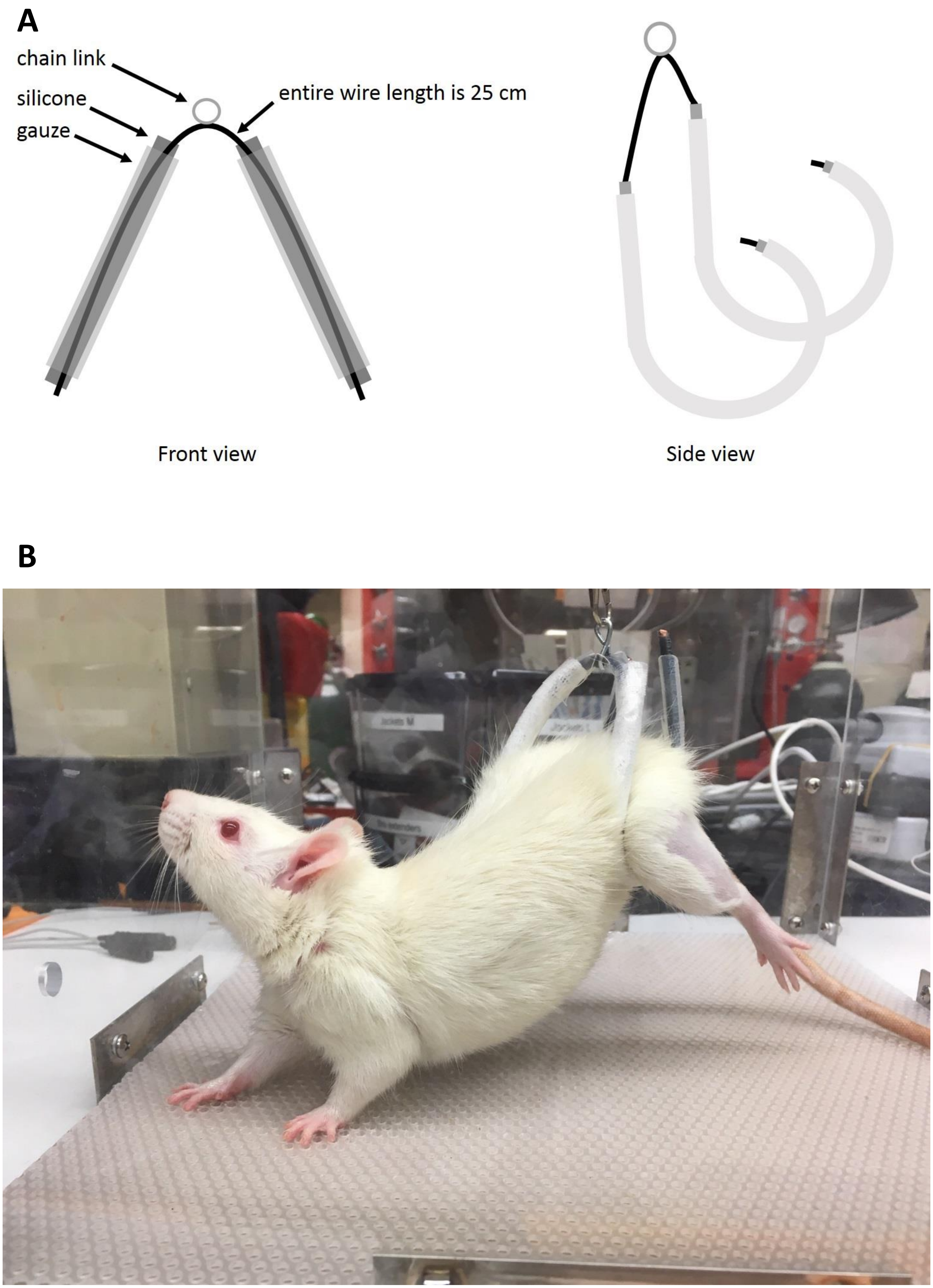
Figure 1 [Click here to access/download;Figure;Figure 1.pdf]

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Front view
Side view
B


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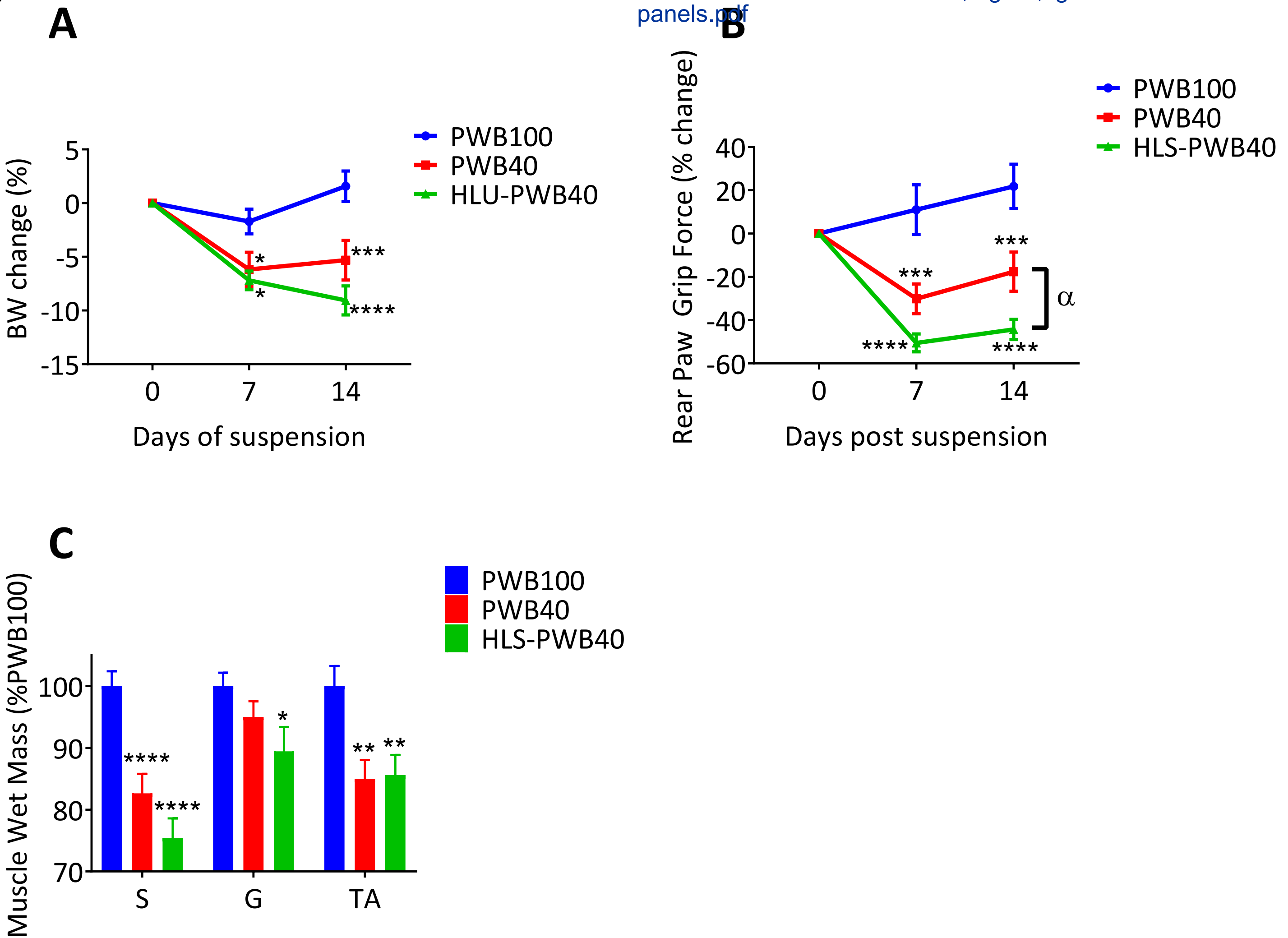
Figure 4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-5
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*
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Days of suspension
B
PWB100
PWB40
HLS-PWB40
Rear Paw Grip Force (% change)
40
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-20
-40
-60
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C
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HLS-PWB40
Muscle Wet Mass (%PWB100)
100
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Name of Material/ Equipment	Company	Catalog Number
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2 Custom design plexiglass walls	P&K Custom Acrylics Inc.	N/A
3M Transpore Surgical Tape	Fisher Scientific	18-999-380
Accessory Grasping Bar Rat	Harvard Apparatus	76-0479
Analytical Scale	Fisher Scientific	01-920-251
Animal Scale	ZIEIS by Amazon	N/A
Back Bra Extenders	Luzen by Amazon	N/A
Digital Force Gage	Wagner Instruments	DFE2-010
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Rat Tether Jacket - Large	Braintree Scientific	RJ L
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Stainless Steel Chains	Super Lover by Amazon	N/A

Comments/Description
100 ft solid building wire with THHN wire type and 10 AWG wire size, black
2 clear plexiglass custom wall 3/16" tick, width 12 3/16", height 18 13/16", 1 rounded slot 0.25 in of diameter located at the center t
Transpore Surgical Tape
Accessory grasping bar rat, front or hind paws
OHAUS Adventurer Analytic Balance
70 lb capacity digital scale big top 11.5" x 9.3" dura platform z-seal 110V adapter 0.5 ounce accuracy
17 pcs 2 hook 3 rows assorted random color women spacing bra clip extender strap
50 N Capacity Digital Grip Force Meter Chatillon DFE II
Non-sterile Cotton Gauze Sponges
PaxCoo 100 pcs metal swivel lanyard snap hook with key rings
Pandahall 100 pcs grade A stainless steel lobster claw clasps 13x8mm
Rodent Jacket
Rodent Jacket
SPX-50 Silicone Tubing
4.5m 15FT stainless steel cable chain link in bulk 6x8mm

op of the wall

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Title of Article: Mimicking a space mission to Mars using hindlimb suspension and partial weight bearing in rats

Author(s): Marie Mortreux, Daniela Riveros, Mary L. Bouxsein, Seward B. Rutkove

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CORRESPONDING AUTHOR:

Name: Marie MORTREUX
Department: Neurology
Institution: Harvard Medical School - Beth Israel Deaconess Medical Center
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January 8, 2019

To the Editorial office of Journal of Visualized Experiments:

We deeply appreciate your consideration of the manuscript entitled: "Mimicking a space mission to mars using hindlimb suspension and partial weight bearing in rats" for publication in the Journal of Visualized Experiments.
The comments of the Reviewers are extremely appreciated and helpful in improving this research article. We have addressed their suggestions individually. The comments are summarized here in black while our responses are presented in **bold and blue**.

Sincerely,
Marie Mortreux, PhD
Harvard Medical School – Beth Israel Deaconess Medical Center

Editorial comments:
Changes to be made by the author(s) regarding the manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
2. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
3. 1.1: Please specify the age, sex, and strain of rat.
4. 1.2: Please mention how proper anesthetization is confirmed.
5. 1.3: Is the pelvic harness custom made or purchased?
6. Line 156: Please use a superscripted number for the in-text reference (Mortreux 2018).
7. References: Please do not abbreviate journal titles.
8. Table of Materials: Please sort the items in alphabetical order according to the name of material/equipment.

We thank the Editor for their comments. The cited points have been addressed in the revised version of the manuscript

Reviewers' comments:

Reviewer #2:
Manuscript Summary:
This is a very interesting technical paper that describes a method to replicate the unloading conditions of partial gravity on the musculoskeletal system. The approach is to physically life all four limbs off the ground equally using a harness around the thorax of the animal until loading is decreased to a fraction of gravity. In the example presented here, this number was 40% loading approximately equivalent to the loading conditions on Mars. This is an improvement on the commonly -used hind limb suspension model because of the lack of fluid shifts, and likely to be less traumatic for the animals. With this model, the authors can look at the effects of (all 4) limb unloading on other elements of the musculoskeletal system such as cartilage, tendon and bone.

Major Concerns:
None.

Minor Concerns:
-Do the rats exhibit reduced activity around the cage with PWB? This could explain the reduced muscle mass and grip strength.
While the cage design is rudimentary since it has no enrichment, animals are still able to move and engage in locomotor activity. This activity is reduced compared to what is observable in standard cages, this is why all of our controls are housed in the same environment. However, locomotor activity has not been quantified in this custom-made housing at the moment.
While the authors allude to the normal behavior of the suspended rats (discussion, para 2), this should be more clearly stated.
Also, was the feeding behavior monitored? Less food intake could result in less overall mass and muscle loss.
Food intake was monitored daily for all animals and no differences were seen amongst the groups.
-line 203: 'claps' should be 'clasp'.
This spelling mistake has been addressed.
-line 264: It is not clear what '.........to walk with more or less difficulty immediately after.......' means. Is it more difficulty, or less difficulty?

This misunderstanding has been addressed.

Reviewer #3:
Manuscript Summary:
This manuscript describes a ground based analogue for partial weight bearing models in rats. As described in the manuscript, PWB may be of great interests to the science community and can have potential to be established as a new method.

Major Concerns:
1. To be established as a model, there needs to be more extensive analyses on other outcome measures besides what's described in the current paper. It's recommended that these additional information be compared to that of reported by Globus et al., Morey-Holton et al, Wronski et al., and the more recent body of literature.
We appreciate the reviewer's comment. This method paper aims at introducing a multiple-gravity levels model that will be suitable for a wide-range of analyses. We previously established and described the PWB model in rats using both bone and muscle outcomes (Mortreux et al., Journal of Applied Physiology, 2018). We hope that this method, which allows for the effective mimicking of a space mission will be used by researchers in different fields and put in context with the recent body of literature.
-There is no measure of stress in the animals. This can be done via survival blood draw and at the time of euthanasia by looking at corticosterone levels in blood or other stress markers in the blood, as well as wet mass and histology of adrenal glands. In fact, the continuous loss of body mass is a clear sign of chronic stress in the animals. There is a large body of literature that shows rats regain body mass after the initial loss during the first 5-7 days.
We agree with the reviewer's comments. While we assessed stress in a widely used qualitative way using grooming, behavior and food intake, we are planning a study to evaluate this in the near future. To address this concern in this manuscript, we have added this limitation to the discussion.
-This contraption has the potential to limit the blood flow to hindlimbs. The authors have not reported any analyses on blood flow or health of internal organs in the groin region.
Qualitative observations did not highlight blood flow problems in this experiment nor in our previous work or in the initial report of the approach, as described by Chowdhury, et al. However, we are currently scheduling a study assessing blood flow both in the hind limbs and the tail to quantify this phenomenon. Internal organs looked normal when tissues were harvested and the groin area was being monitored daily to ensure that there was no abrasion. The proper maintenance and padding of the pelvic harness is designed to provide comfort and minimize issues in the inguinal region. We have now included this concern to the discussion as well.
-For this to be established as a model for musculoskeletal disuse, analyzing and presenting bone data (uCT and histomorphometry) is essential, which is not included in this paper. This should include hindlimbs (femur and tibia) as well as forelimbs (for PWB) to be compared across the groups to validate the model.
We appreciate the reviewer's comment. However, this study aims at introducing a model for exploring the gravity as a continuum and the musculoskeletal alterations occurring during PWB have been previously published. Our manuscript does not reflect the totality of the experiments that can be performed using this model in the future. We have added a comment to the discussion to address this point.
-The images of animals that are present in the manuscript show an unnatural curvature of the spine. There needs to be careful analysis of the spinal column (by uCT and/or FEA) to investigate whether there is additional tension or compression in any region of the spinal column.
MicroCT analysis of the spine has not yet be performed but we will collect data in the future to address this concern. No specific change has been made to address this comment.

2. In this model, the animals are limited in their mobility due to taped rod, which is a huge disadvantage and may cause stress to the animals.
While this would be the case for mice, we previously demonstrated that the center location of the suspension device does not significantly prevent the animals from moving, resting, grooming, and accessing food and water. While we understand the reviewer's concern, previous data does not support this. Again, we plan to study the stress issues as part of future work.
3. Animals were not provided with any type of enrichment (chew bones, nestlet, tisse, etc.)
We agree with the reviewer and we will use enrichment in our next experiments.

Minor Concerns:
1. Describe the frequency of titration for PWB and the justification on how that was selected.
Titration of the PWB was realized daily. While creating the model, we were checking twice a day, however, our extremely low coefficient of variations demonstrated that there was no need. A titration once daily, at the beginning of the light phase, allows us to take into account potential weight gains or losses that could have happened overnight.
2. The authors only cite PWB literature in mice that is internal to their group while there are other bodies of literature that have performed experiments (Macias et al, Swift et al.) and improved on the model initially developed by Wagner et al.
We thank the reviewer for their comments. We have now reviewed other bodies of literature and included the following reference Swift, J.M. et al., Partial Weight Bearing Does Not Prevent Musculoskeletal Losses Associated with Disuse. Medicine & Science in Sports & Exercise. 45 (11), 2052-60.
3. There is no clear mention of the study design and why the groups are n=9 and n=12 (i.e. attrition rate).
10 animals were exposed to HLU + PWB40 and one was removed, resulting in n=9 for the data collected. Results were then put into context using the ones we previously published in the Journal of Applied Physiology, where the n for each group (PWB100 and PWB40) was 11-10 per group, hence the discrepancy. We apologize for the misspelling of n=12, which has now been corrected.
4. To conduct a proper study, animals should be pair fed across groups.
We agree with the reviewer as it is envisioned for our next experiments and has been done in the mouse model as well.
5. For this to be helpful to other scientists, it's recommended to include drawings of the hindlimb contraption and the jacket design.
The jacket is purchased while the pelvic harness is custom-made. An illustration has been added to improve clarity (figure 2A).

Reviewer #4:
Manuscript Summary:
This paper describes a (not entirely) novel method for achieving partial weightbearing in rats, an adaptation of this group's previously described method for mice (Wagner et al., 2010). The pelvic harness certainly does make the transition from full unloading (hindlimb suspension) to PWB more convenient. Given that many users of these techniques are interested in bone outcomes, the paper would be considerably stronger (and of more general interest) if bone outcomes would be validated. Additionally, a bit more discussion of the finer points of the methodology would be helpful.

Major Concerns:
1. This paper would be of higher interest if efficacy of protocol could be demonstrated for bone outcomes in addition to muscle, as they did in their 2018 JAP paper (with one outcomes using pQCT). It is far easier to demonstrate efficacy with muscle, the more plastic tissue which changes far more rapidly than bone.

We appreciate the reviewer's comment. While our first paper describing the method used both bone and muscle outcomes as validation tools, this article only aims at presenting a model to mimic a space mission by allowing for multiple and successive gravity-levels. The model described here is however entirely suitable for other musculoskeletal analyses including tendon, cartilage, and bone measurements. You can see response to reviewer #3.

2. The previous iteration of this method generated by this research group used a tension spring to connect the PWB harness to the overhead rod; why was this not used with rats? How does the switch from a spring to simply adjusting the length of the suspension chain impact on the efficacy of the model? On the consistency of loading?

As discussed in our initial work, we adapted the design from mice to rats by removing the spring as it provided a steadier partial unloading of the animals while not restricting their movements or access to food and water. In our initial work, we demonstrated that the chain-based suspension design improved the steadiness of the PWB level, therefore increasing the accuracy of the model. The consistency of the partial unloading was assessed daily and does not exceed 1.20% variation in average for rats exposed to PWB40 (Mortreux et al., 2018).

3. For this method to be effective in hindlimb suspension as well as partial weightbearing, it's essential to minimize the ability of the hind feet to contact the side walls of the cage and push off those. How was this achieved?

When first secured in their pelvic harnesses, animals are monitored to make sure that their hindlimbs cannot touch the ground. Afterwards, the centered suspension device does not allow them to reach the walls and to use them to "re-load" themselves.

4. Line 242 brings up a major issue: in the transition from full unloading to PWB, the loss of muscle function post full unloading "may result in pain"… this could be a major issue for IACUC panels in reviewing this method. What is the evidence the authors have for these rats experiencing pain? For how long do their rats exhibit awkward gait or lethargy or (more importantly) agitation or outright squeaking? "Reloading injury" is well-known among muscle biologists using hindlimb unloading, but shares common mechanism with eccentric exercise-induced muscle soreness in humans.

We appreciate the reviewer's comments about animal pain and we take animal welfare extremely seriously. All of our protocols have been approved by the IACUC. We do not have evidence of pain *per se*, we corrected the sentence to accurately reflect our observations. Indeed, immediately after reloading the animals from HLU to PWB40, some rats did exhibit awkward gait. However, none of them displayed any signs of lethargy, agitation, or squeaking. We do not think this transient gait modification could be the result of a reloading injury but is mostly related to the transition from bipedal walking using only the front limbs to quadrupedal walking.

5. Figure 1 illustration shows what appears to be tape immobilizing the chain to be attached to the animal's harness--- this would limit access of the animal to all areas of the cage including to water/food.

As we previously described in our initial work, the suspension device is immobilized at the center of the cage but does not prevent the access to food and water. It does slightly restrict the access to the edges of the corners without preventing the animals to move / walk in their cage.

6. Given that this journal is focused on methodology, more discussion should be included on certain points:

a. Is the pelvic harness tolerated equally well by female and male rats? Is there a limit on rat size (minimum and maximum weights)?

This is an extremely interesting remark as we aim at investigating the sex-differences in our PWB model. However, we have not realized this experiment on female rats at the moment. We have worked with animals ranging from 300-550 grams and all were well tolerating the harness. Additionally, our harnesses are manually positioned onto the rats, therefore ensuring a proper fit.

b. what are the pro's and con's of anesthetizing rats to apply the PWB jacket and harness?

Anesthetizing animals for the first installation of the PWB jacket and harness is helpful to ensure the best fit and to familiarize new users. However, with some practice, the ability to perform it on awake animals allows for fine daily tuning and is our standard method in the laboratory.

c. For the few rats who "escaped", how did they do so? Is this problem specific to the pelvic harness? What tips are there for minimizing escapes?
Rats who "escaped" usually did so by extensively moving around while trying to chew their harnesses. As we only used a pelvic harness for the rat PWB model, we can not compare our statistics with a standard tail suspension in the same conditions. In order to minimize escapes, it is important to check the condition of the harness daily when assessing the PWB or well-being of the rats, making sure that any damaged harness is fixed or replaced. However, the escapes we observed remain extremely marginal and did not present a significant problem during our experiments.

Minor Concerns:
1. There is ambiguity in the abstract: 'musculoskeletal' is used twice but the middle of the abstract refers only to muscle. Since this paper presents no outcome data on bone (nor is it likely that detectable bone changes would occur within the 14 d of this protocol), all references to 'musculoskeletal' should be removed.
We agree with the reviewer's comment that our results section is focused on muscle changes and removed some of the references to "musculoskeletal" in the text. However, we preserved some of them in order to put our work into the larger context of a ground-based analogue for space-induced alterations.
2. Give more information on the rats used to generate these data: sex and age and strain of rats.
This has been omitted and was added to the protocol.
3. Instructions for grip force: for 3.3, given that you are assessing hindlimb grip strength, shouldn't it be "pull the rat straight forward"?
Grip force for both front and hind limbs is given by a force transducer attached to a pull-bar. The device is able to record in both directions, however, the animals can only be pulled away from the transducer. We hope the video of this step will provide further explanation.
4. Is there any food intake data to help explain the body weight changes illustrated in Figure 4A?
We did measure food intake daily and did not notice any significant differences.
5. Figures 4A and 4B: "post" should be removed from the x-axis label.
This has been corrected in figure 3.
6. Line 248: why would there be edema in the rear paws if they are elevated?
While the rear paws are elevated, animals keep their hind limbs down once secured in the harness, therefore decreasing the venous return from the hind limbs and creating slight edema.
7. Lines 290-291: significant correlation for what variable?
We apologize for the omission of this detail. The correlation was established based on the soleus wet mass, and the manuscript has been amended.
8. Line 305: explain what sequential-reloading is--- gradually increasing % weightbearing?
Sequential reloading would indeed be gradually increasing the weight-bearing of our animals to minimize stress and reloading injury.

January 15, 2019

To the Editor:

We would like to thank you for the comments made regarding our article. In this document, we will address each comment separately.

The editor has formatted the manuscript to match the journal's style. Please retain the same.

We thank the editor for the formatting of the manuscript and have not made any modifications

Figure 4: Please line up the panels better. Some panels are off-set.

The panels as well as the letters have been realigned.

Protocol: Please add a section to describe how to obtain the muscle mass as results have been presented. Please note that this section does not have to be filmed. The authors can also add references to published material specifying how to perform the protocol action.

A protocol section numbered 4. And entitled recording of muscle wet mass has been added to the non-filmed part of the protocol.

Please ensure that each reference cited in text appears in the reference list, and each entry in the reference list is cited in text.

We apologize for the mistake. References 17-19 have been removed from the text and have now been removed from the reference list. Additionally, the mistake “(ref)” has been corrected by the appropriate reference number.

Please review the figure legend to ensure that descriptions of abbreviations added by the editor are correct.

We have reviewed the abbreviations added by the editor and they are correct. We only placed the PWB100 group before the experimental ones in the legend.