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RESEARCH ARTICLE

Fluid shift versus body size: changes of hematological parameters and body fluid volume in hindlimb-unloaded mice, rats and rabbits

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ABSTRACT

The cardiovascular system is adapted to gravity, and reactions to the loss of gravity in space are presumably dependent on body size. The dependence of hematological parameters and body fluid volume on simulated microgravity have never been studied as an allometric function before. Thus, we estimated red blood cell (RBC), blood and extracellular fluid volume in hindlimb-unloaded (HLU) or control (attached) mice, rats and rabbits. RBC decrease was found to be size independent, and the allometric dependency for RBC loss in HLU and control animals shared a common power (−0.054±0.008) but a different Y_0 coefficient (8.66±0.40 and 10.73±0.49, respectively, $P<0.05$). Blood volume in HLU animals was unchanged compared with that of controls, disregarding body size. The allometric dependency of interstitial fluid volume in HLU and control mice shared Y_0 (1.02±0.09) but had different powers N (0.708±0.017 and 0.648±0.016, respectively, $P<0.05$), indicating that the interstitial fluid volume increase during hindlimb unloading is more pronounced in larger animals. Our data underscore the importance of size-independent mechanisms of cardiovascular adaptation to weightlessness. Despite the fact that the use of mice hampers application of a straightforward translational approach, this species is useful for gravitational biology as a tool to investigate size-independent mechanisms of mammalian adaptation to microgravity.

KEY WORDS: Simulated microgravity, Erythrocytes, Blood volume, Interstitial fluid volume, Allometry

INTRODUCTION

When a human body enters microgravity during a space flight, gravity ceases to pull blood to the lower part of the body, causing blood and other body fluids to redistribute. This fluid shift is manifest in the well-documented decrease of leg volume (Fortrat et al., 2017; Thornton et al., 1987), putative increase in intracranial pressure (Zhang and Hargens, 2018) and face puffing (Kirsch et al., 1993). Blood redistribution is thought to be the key initiating event for cardiovascular adaptation to weightlessness (Watenpaugh and Hargens, 1996), followed by a decrease in blood volume (Johnson, 1979), due to loss of plasma (Smith et al., 1997) and erythrocytes (Ivanova et al., 2007; Noskov et al., 1991; Poliakov et al., 1998; Tavassoli, 1982), an increase of stroke volume (Norsk et al., 2015), and alterations of heart rate (Baevsky et al., 1997; Karemaker and Berecki-Gisolf, 2009; Verheyden et al., 2009) and central blood pressure regulation (Baevsky et al., 2007; Di Rienzo et al., 2008; Fritsch et al., 1992; Morita et al., 2016; Pagani et al., 2009). While these changes of cardiovascular functions pose no threat during the space flight, after landing the cardiovascular system is no longer capable to sustain normal blood pressure while standing (Buckey et al., 1996; Kotovskaya and Koloteva, 2016; Lee et al., 2015) or with other loads (Fu et al., 2004; Levine et al., 1996). Time and medical aid suffice to overcome the post-flight cardiovascular disadaptation on Earth (Laughlin et al., 2015; Payne et al., 2007; Vasilyeva and Bogomolov, 1991), but after landing on other planets both might be limited and thus disadaptation of the cardiovascular system might restrict the ability of cosmonauts/astronauts to work in this busy period.

While blood redistribution seems sufficient to initiate cardiovascular adaptation to microgravity in upright humans, it is far less clear what triggers cardiovascular changes in space-flown laboratory rodents. In rats and, more recently, mice, alterations of blood composition (Serova et al., 1993; Udden et al., 1995), morphological and functional remodeling of resistive arteries (Behnke et al., 2008; Sofronova et al., 2015; Stabley et al., 2012; Taylor et al., 2013) and veins (Behnke et al., 2013), and heart rate (Fuller et al., 2003) and baroreflex sensitivity (Waki et al., 2005) have all been reported after space flights of various durations. Recently, we have extended observations of cardiovascular disadaptation in rodents to space-flown mice using implantable telemetry in the 30 day Bion-M1 biosatellite space flight and during the post-flight recovery (Andreev-Andrievskiy et al., 2017). In summary, ample experimental data support the existence of cardiovascular adaptation to space flight in small quadruped mammals with minimal, if any, hydrostatic pressure gradient.

The model of hindlimb unloading, originally developed by Ilin and Novikov (1980) and popularized by Morey-Holton (Morey-Holton and Globus, 2002), has been repeatedly applied to on-ground studies of cardiovascular adaptation to microgravity. The hindlimb-unloaded rats and mice display vascular remodeling (Behnke et al., 2008; De Salvatore et al., 2004; Summers et al., 2008), hematological changes (Dunn et al., 1985; Ryou, 2012) and alterations of blood pressure and/or heart rate (Powers and Bernstein, 2004; Tarasova et al., 2001; Tsvirkun et al., 2012; Zhang et al., 2008), along with other signs of cardiovascular adaptation (Bouzeghrane et al., 1996; Brizzee and Walker, 1990; Chew and Segal, 1997; Fagette et al., 1995; Moffitt et al., 1998). In relation to fluid shifts, the increase of hydrostatic pressure in the neck tissues (Hargens et al., 1984) and intracranial pressure was reported in hindlimb-unloaded rats (Krasnov et al., 2005; Maurel et al., 1996), although measurement of blood and interstitial fluid volumes produced ambiguous data (Bouzeghrane et al., 1996; Chew and Segal, 1997; Deever et al., 2002). In larger animals (rabbits), changes of intracranial pressure have been reported during unloading (Tatebayashi et al., 2003) and cerebral perfusion is enhanced during short-term microgravity in a parabolic flight (Florence et al., 1998).

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Despite the variability of the results with the species used, unloading duration and other experimental parameters, hindlimb unloading, or the 'suspension' model, proved to be fruitful over the years and still remains virtually the only suitable model for investigation of on-ground microgravity effects in animals.

It is well known that 'sensitivity' to gravity depends on body size. Non-linear increases in bone mass in order to support the increasing body weight that limits the size of terrestrial animals (Alexander, 1985) is a nice illustration of this principal. In contrast, mice can survive roughly tenfold higher acceleration than humans (Chae, 1975) and, as an extension of this relationship clearly seen in routine laboratory practice, cells are perfectly viable after centrifugation at hundreds of ***g***. When applied to the other side of the gravity continuum, i.e. microgravity, smaller animals can be expected to be less responsive to the loss of gravity during a space flight or on-ground modeling. This notion complements well the concept of fluid shifts. Apparently, the larger the hydrostatic pressure, the greater the effects of its elimination should be, and vice versa. Although the fluid shifts have been investigated in a series of brilliant studies by Hargens and colleagues using several larger species (Hargens et al., 1987; Lillywhite et al., 1996), they have never been systematically analyzed as a function of body size at the other end of the axis, in smaller mammals. Because of their minute body size, the hydrostatic pressure gradient in mice cannot exceed ∼2 mmHg while in the normal posture and ∼5 mmHg when a mouse rears; thus, it could be expected that the changes of body fluid volume and hematological parameters would be less pronounced in this species than in larger animals.

Our study was aimed at investigating the dependence of microgravity-induced fluid shifts on body size in smaller laboratory mammals as an allometric function. To this aim, we measured blood and interstitial fluid volume and hematological parameters in hindlimb-unloaded mice, rats and rabbits. We found that in the body size range studied, only the interstitial fluid volume increase under simulated microgravity conditions is proportional to body size, while blood volume reduction and red blood loss are similar among the three species. Thus, we conclude that the fluid shift alone cannot explain the observed changes of body fluid volume and hematological reactions and underscore the importance of other, size-independent factors.

MATERIALS AND METHODS

Animals and housing

Male BALB/C mice (n=40, 25–30 g), Wistar rats (n=39, 250–350 g) and New Zealand white rabbits (n=10, 2.5–3.5 kg) were used in this study. Mice and rats were purchased from the Stolbovaya branch of the Scientific Center for Biomedical Technologies of the Federal Medical and Biological Agency and rabbits from a regional rabbit breeding center (LLC KrolInfo, Moscow region, Russia). Animals arrived at least 2 weeks before the experiment for adaptation.

Prior to hindlimb unloading, mice and rats were housed in groups of 3–5 individuals each, in standard plastic cages (floor area 500 cm^2 for mice, 800 cm^2 for rats) with wood chip bedding (JRS, Rosenberg, Germany) and appropriate environmental enrichment (nesting chambers and nesting material for mice, wooden playthings for rats). Rabbits were housed in individual cages (floor area 1.2 m^2, height 120 cm), with wooden slatted decking. Standard rodent (Assortiment-Agro, Moscow region, Russia) or rabbit (KrolInfo) chow and deionized water or tap water were provided, respectively, to mice/rats and rabbits *ad libitum*. During hindlimb suspension, animals were housed individually. All efforts were made to enable communication with conspecifics for individually housed animals. Temperature was maintained at 20–24°C for mice and rats and 16–22°C for rabbits, humidity was 30–70%, and the light cycle was maintained at 12 h.

The provisions of the European convention for the protection of vertebrate animals used for experimental and other scientific purposes (1986) were carefully followed.

Experimental design

Two experiments were performed. In experiment 1, hematological parameters were monitored longitudinally in the same animals for the 7 days of hindlimb suspension. In experiment 2, blood and interstitial fluid volume was measured on day 3 of unloading and compared with values in control animals.

Hematological parameters during hindlimb unloading

In order to follow the dynamics of hematological parameters in mice (n=10), rats (n=10) and rabbits (n=5), blood samples were collected repeatedly from the same animal the day before (−1), just prior to (0), at 10 min, 1, 6, 12 h, 1, 2, 3, 7 days of unloading and 10 min and 1 h after reloading. To facilitate blood collection in mice and rats, jugular catheters were implanted 2 days prior to experiments; in rabbits, blood was collected via lateral ear vein puncture. The collected blood samples were analyzed using an automated hemocytometer.

Blood and interstitial fluid volume in hindlimb-unloaded animals

Blood volume and interstitial fluid volume was estimated, respectively, from FITC-conjugated dextran and inulin distribution volume. To calculate distribution volume, pharmacokinetic curves were obtained after single intravenous administration of the probes. For mice and rats, two groups of animals were used, the hindlimb-unloaded (HLU) and the control group; control animals were attached without actual unloading. The number of animals was 7–9 for each of the combinations of species, probe and group. In order to reduce the number of animals used for the study, rabbits were used in a cross-over design. Pharmacokinetic data for FITC-dextran and FITC-inulin were collected during hindlimb unloading and attachment in the same animal (5 rabbits per probe) with 3 weeks of recovery between the experiments.

Hindlimb unloading

Hindlimb unloading of mice and rats was performed as described by Morey-Holton and Globus (2002) with the modification of Ferreira et al. (2011) used for mice. Briefly, in mice a stainless steel ring was implanted between the third and fourth tail vertebra under general and local anesthesia, simultaneously with jugular catheter implantation (described below). In rats, a stainless steel hook was attached with Omniplast adhesive tape (Hartmann, Heidenheim, Germany) at the base of the tail after catheter implantation. Once the animals had recovered from anesthesia, they were placed individually into plastic suspension cages with a wire mesh floor (floor area 600 cm^2). The day after the operation, mice and rats were attached to the suspension string (without unloading). Thus, animals were gradually habituated to the unloading apparatus over 2 days. On the third day, HLU animals were hindlimb unloaded for 7 days at approximately 30 deg between the body axis and the cage floor plane, while the control mice and rats were left attached without unloading. Nesting material and a solid place for animals to rest were provided throughout housing in the suspension cages.

For hindlimb unloading of rabbits, metal cages 1.2×1.1×1.1 m and nylon anatomic body harnesses were used. The timeline of the procedures was similar to that for mice and rats. First, rabbits were harnessed and placed into the suspension cages for 1 day. The next

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day they were attached to the suspension apparatus, which allowed free movement of the animals across the cage. After these 2 days of adaptation, rabbits were either unloaded in HLU experiments or left attached in control runs.

Blood collection and handling

In mice and rats, blood was collected using jugular catheters in both experiments. Jugular catheters were implanted 2 days prior to unloading. Briefly, animals were anesthetized with a combination of zolazepam and tiletamin (15–20 mg kg^{-1} each) supplemented with xylazine (5 mg kg^{-1}) administered intraperitoneally. The right jugular vein was accessed through a neck skin incision and suspended on ligatures. Through a puncture in the vein wall, plastic catheters (PE 10, 0.6 mm outer diameter) were advanced into the upper vena cava so that the tip was adjacent to the heart (10–12 mm in mice, 22–25 mm in rats) and secured in place with ligatures and medical grade acrylic glue. The catheters were exteriorized in the scapular area and the incisions stitched with absorbable 5-0 or 4-0 sutures. Animals received appropriate veterinary care after surgery and, in our hands, easily recovered with minimal or no weight loss. The catheters were filled with heparinized (100 U ml^{-1}) sterile saline and flushed daily. Blood samples of ∼12 and 50–60 µl per time point were obtained from mice and rats correspondingly using a glass Hamilton syringe and PE tubing.

For blood collection in rabbits, a simple venipuncture of the lateral ear vein was used in experiment 1, while peripheral catheters (Troge, Hamburg, Germany) were implanted for blood collection in experiment 2 using a standard technique. Blood samples were 50–100 µl in both experiments.

All measures were taken to prevent blood dilution with the catheter filling fluid. The withdrawn blood volume was replaced with sterile saline (0.9% NaCl).

Hematological measurements

For standard hematological calculations, blood samples were stabilized with K2-EDTA and analyzed no longer than 2 h after collection using an automated hemocytometer 1280vet (Dixion, Moscow, Russia) and control samples (Streck, La Vista, NE, USA) for quality control.

Blood and interstitial fluid measurements

Blood and interstitial fluid volume was estimated from the distribution volume of FITC-labeled dextran (MW=150 kDa) and inulin (MW=4 kDa), respectively. Both probes were purchased from TdB Consultancy AB (Uppsala, Sweden) and purified from unconjugated FITC by dialysis with 1 kDa membrane vials (Orange Scientific, Braine-l'Alleud, Belgium) against 1 l water for 24 h, protected from light. Because of its large molecular weight, FITC-dextran is not subject to extravasation, unlike FITC-inulin, which distributes between the blood and the interstitial fluid. These properties of the probes determine their monoexponential and biexponential pharmacokinetics upon intravenous administration (Fig. 1). The pharmacokinetic analysis of FITC-dextran and FITC-inulin concentration in blood (unlike measurement of dilution at an arbitrary selected single time point) provides an accurate estimate of blood and interstitial fluid volume.

FITC-dextran was injected at a dose of 2 mg kg^{-1} into mice and 1 mg kg^{-1} into rats and rabbits. We specifically sought to use the minimal doses that generate detectable plasma fluorescence in order to reduce volume expansion due to high dextran osmolarity. For FITC-inulin, the doses in mice, rats and rabbits were 25, 5 and 2 mg kg^{-1}, respectively. The difference in doses used for these three species was governed by the need to reduce blood sample volume, and the consequent requirement for greater dilution of the smaller blood samples.

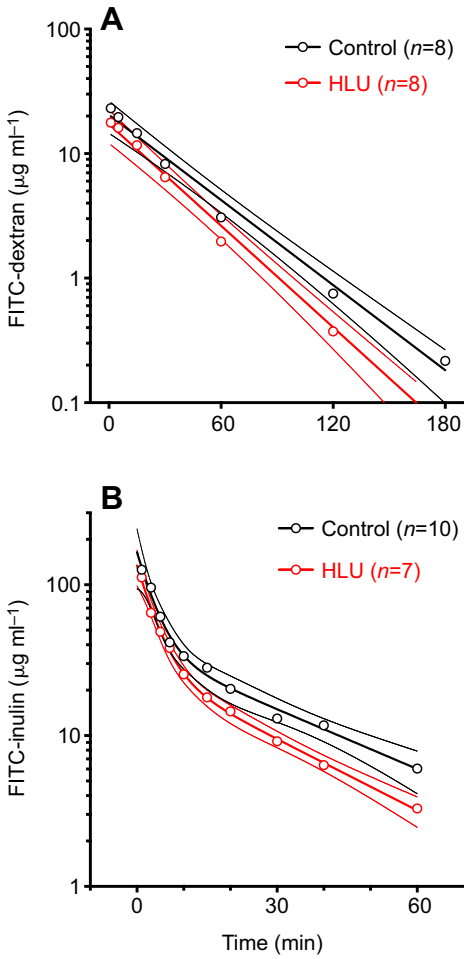

Fig. 1. Blood and interstitial fluid measurements. Pharmacokinetic curves of FITC-dextran (A) and FITC-inulin (B) in hindlimb-unloaded (HLU) or attached (control) mice. High molecular weight (M=150 kDa) FITC-dextran does not extravasate, as reflected by monoexponential pharmacokinetics, and its distribution volume (V_{ss}) was used as an estimate of blood volume. FITC-inulin has a smaller molecular weight (M=4 kDa) and distributes between the blood and interstitial fluid – hence the biexponential pharmacokinetic curves – and its V_{ss} was used to estimate interstitial fluid volume. Circles represent experimental data, solid lines show the regression curve and dotted lines show the 95% confidence interval.

Blood samples were collected 1, 5, 15, 30, 45, 60, 120, 180, 240 and 360 min after FITC-dextran administration and 1, 3, 5, 7, 10, 15, 20, 30, 40, 60 and 120 min after FITC-inulin injection repeatedly from the same animal. Plasma was separated by centrifugation at 2000 rcf for 10 min (hematological capillaries were used for centrifugation of smaller samples), mixed with 0.5 mol l^{-1} Hepes 9:1 v:v and frozen at −18 to −22°C, protected from light till subsequent analysis.

The fluorescence of these samples was quantified using Anthos Zenyth 3100 (Biochrom, Cambourne, Cambridge, UK) microplate reader with 485 nm excitation and 595 nm detection wavelengths. Standard curves were obtained using donor blood from the corresponding species, quantified simultaneously with experimental samples and used to transform fluorescence intensity into concentration.

Data analysis

Pharmacokinetic data were analyzed using WinNonLin (v.7.0, Certara, Princeton, NJ, USA) software and a one-compartment model for FITC-dextran (Eqn 1) and two-compartment model (Eqn 2) for FITC-inulin; distribution volume was calculated as V_{ss}:

$$C = Ae^{-\alpha t}, \quad (1)$$

$$C = Ae^{-\alpha t} + Be^{-\beta t}. \quad (2)$$

In order to investigate the dependency of hematological parameters and body fluid volume on body size, values were plotted against body mass (M_b) of the corresponding animal. The parameters of allometric equations (Eqn 3) were calculated using Prism (v.6.0, GraphPad, La Jolla, CA, USA). The hypothesis that the equations for control and HLU animals have different quotients Y_0 and N was tested using a nested F-test with the same software:

$$Y = Y_0 M_b^N. \quad (3)$$

The body mass-corrected values were analyzed with two-way ANOVA followed with Sidak's post-test using Prism (v.6.0, GraphPad). The differences were considered significant at $P<0.05$. Unless indicated otherwise, the data are presented as means±s.e.m.

RESULTS

Hematological parameters during hindlimb unloading

As presented in Fig. 2, red blood cell (RBC) count was, as expected, highest in mice and lowest in rabbits, and intermediate in rats. In all the three species, RBC count decreased during hindlimb unloading; the first significant decrease in mice (by 7.6±3.6% compared with baseline) was observed as soon as 6 h after unloading, whereas in rats, RBC count was decreased compared with baseline values starting from the third day of unloading, and in rabbits, from the second day of unloading. Generally, RBC count decrease progressed slowly over the first 3 days and plateaued by the end of the 7 day suspension period. It should be noted that in rats and rabbits, RBC dynamics followed a more complex pattern with two phases of RBC decrease: just after and approximately 2 days after unloading. Immediately after reloading, a moderate rise in RBC count was observed in all three species.

As shown in Fig. 3A, RBC count was progressively smaller in mice, rats and rabbits ($F_{2,21}$=20.21, $P<0.0001$) and decreased with hindlimb unloading ($F_{1,21}$=101.1, $P<0.0001$). When plotted as a function of body mass (Fig. 3B), the dependency of RBC count on body mass during attachment and hindlimb unloading was characterized by a shared power of −0.054±0.008 ($F_{1,44}$=1.54, P=0.2208) rather than different powers (−0.063±0.010 and −0.044±0.011 for control and HLU, respectively). The Y_0 coefficients were, however, markedly different for control (10.73±0.49) and HLU (8.66±0.40) states ($F_{1,44}$=12.48, P=0.0010). Thus, as indicated by a shared slope of RBC dependency on body mass, the hindlimb unloading-induced RBC decrease was relatively independent of body size. The baseline differences in RBC count were compensated by the proportional difference in mean cell volume; thus, the hematocrit was close in the three species studied and, similar to RBC count, decreased uniformly with hindlimb unloading (data not shown).

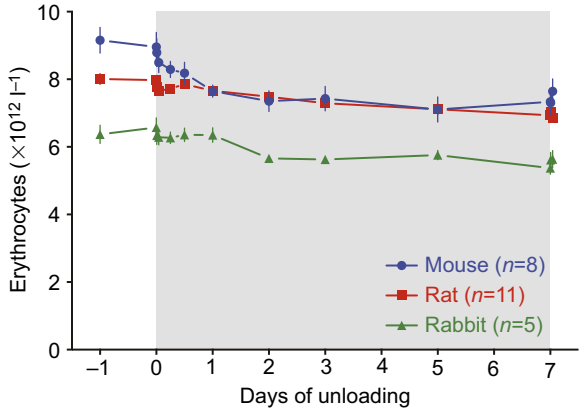

Fig. 2. Erythrocyte count in mice, rats and rabbits during 7 days of hindlimb unloading (gray shading). Data are means±s.e.m. The red blood cell (RBC) count followed similar dynamics in all three species, gradually decreasing over the first 3 days of unloading and plateauing by day 7.

Blood volume during hindlimb unloading

Blood volume was estimated from the distribution volume (V_{ss}) of high molecular weight FITC-dextran. When corrected for body mass (Fig. 3C), blood volume varied with species ($F_{2,38}$=19.22, $P<0.0001$) but was similar in hindlimb-unloaded and control animals ($F_{1,38}$=1.37, P=0.2484). The allometric functions for blood volume in hindlimb-unloaded and control animals (Fig. 3D) were indistinguishable, with Y_0=0.131±0.015 and the power N close to 1 and equal to 0.917±0.020 for both, as preferred to individual quotients ($F_{1,40}$=0.43, P=0.5146 and $F_{1,40}$=0.00, P=0.9609 for Y_0 and N, respectively). Thus, there were no differences in blood volume between the hindlimb-unloaded and control animals, disregarding the size of the animal.

Interstitial volume during hindlimb unloading

Interstitial volume was estimated from FITC-inulin distribution volume. The analysis of variance of the body mass-corrected values (Fig. 3E) revealed that interstitial fluid volume variability depended on the species ($F_{2,35}$=189.2, $P<0.0001$) and hindlimb unloading ($F_{1,35}$=7.68, P=0.0002). It should be mentioned that blood accumulation in the cranial part of the body was quite visible in all hindlimb-unloaded animals, as well as edema in some of the hindlimb-unloaded rabbits, but not rats or mice. The allometric dependency of interstitial fluid volume in HLU and control animals (Fig. 3F) had a shared Y_0 value (1.02±0.09; $F_{1,37}$=1.21, P=0.2795) but different powers (N=0.632±0.030 for control and 0.720±0.014 for HLU individuals; $F_{1,37}$=6.75, P=0.0134). The greater power of the allometric equation for HLU as compared with control animals indicates that the interstitial fluid increase is more pronounced in larger animals.

DISCUSSION

In this study, we addressed the question of whether changes in hematological parameters and body fluid volume during simulated microgravity, as indicators of fluid shift, are dependent on the size of the organism. To this aim, we investigated changes in RBC parameters, and blood and interstitial fluid volume in hindlimb-unloaded mice, rats and rabbits. Using these data, we calculated the allometric equations for the unloaded and control animals and found that RBC count decrease was relatively independent of body size, blood volume does not change during unloading, while interstitial fluid volume expands in hindlimb-unloaded animals and the magnitude of this increase is proportional to body mass. Thus, among the parameters we investigated, only the interstitial fluid volume followed the pattern we anticipated, while the magnitude of other reactions was similar among species of different size.

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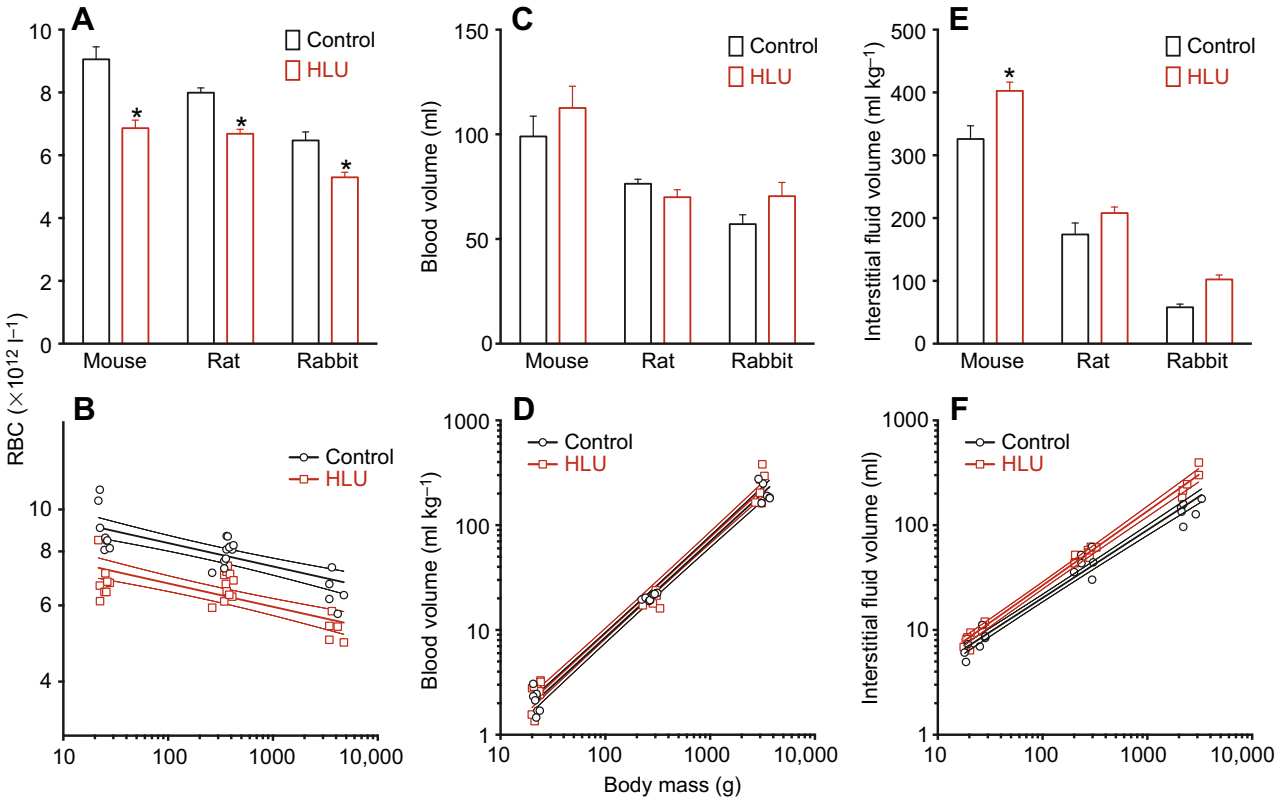

Fig. 3. RBC count and blood and interstitial fluid volume changes during hindlimb unloading. (A) RBC count in hindlimb-unloaded (HLU) or control mice, rats and rabbits and (B) log–log analysis of RBC dependency on body mass. (C) Blood volume corrected for body mass and (D) plotted as a function of body mass. (E) Interstitial fluid volume corrected for body mass and (F) its dependency on body mass. Data are presented as means+s.e.m. in A, C and E. In B, D and F, symbols represent experimental data, solid lines are regression curves and dashed lines are 95% confidence intervals. **P*<0.05, Sidak's test.

Though frequently applied to diverse physiological systems, the scaling approach has seldom been used in gravitational physiology studies (Chae, 1975; Pace et al., 1981). To the best of our knowledge, our report is the first to quantify the effects of simulated microgravity as an allometric function. However, several studies have investigated hematological parameters and body fluid volume, or other fluid shift-related measures in different species, primarily rats and, less frequently, mice or rabbits.

Reduction of RBC volume is a long-established consequence of space flight in humans (Leach and Johnson, 1984; Tavassoli, 1982). It has also been reported in monkeys (Gazenko and Ilyin, 1987) and, somewhat controversially, in rats (Allebban et al., 1996; Kalandarova et al., 1976; Lange et al., 1987; Serova et al., 1993; Udden et al., 1995; Vacek et al., 1982). Isolated measurements of murine hematological parameters produced opposite data (Rizzo et al., 2012), which could, however, be compromised by factors unrelated to microgravity. Unlike real space flight, in on-ground simulation studies with rats, RBC and hematocrit were reported to decrease (Dunn et al., 1985; Nezami et al., 2016) or remain unaltered (Chew and Segal, 1997; Ryou, 2012; Saunders et al., 2002). A reduction of RBC count after hindlimb unloading was also found in ground squirrels, used in research for their ability to hibernate (Hu et al., 2017). Thus, the RBC decrease we observed in three quadruped species is in accordance with some, but not all, of the previous findings. We cannot offer a reasonable explanation for this apparent incongruity between results from different labs, except that age differences might be implicated, as we prefer to use more mature animals rather than 2- to 3-month-old 'teen' rodents. In our hands, the RBC decrease was reproducibly found in tail-suspended mice (Popova et al., 2017); single-point measurement was used in our previous study, unlike repeated sampling utilized in this experiment.

A decrease of blood and interstitial fluid volume is another well-known reaction of the human body to weightlessness (Johnson, 1979; Tavassoli, 1982) that can be reproduced on Earth using antiorthostatic hypokinesia (Morukov et al., 2003; Zorbas et al., 2003) or dry immersion (Chaika and Balakhovskii, 1982; Greenleaf et al., 1977). In monkeys, a drastic 30% decrease in interstitial fluid volume was found following a 7 day Cosmos space flight (Gazenko and Ilyin, 1987). For a smaller species (rats), far less pronounced changes were reported using radio-labeled probes during on-ground simulation (Deever et al., 2002; Somody et al., 1998). Enhanced plasma filtration was found in tail-suspended rats using the blood density difference between the arterial and venous blood (Medvedev et al., 1998) and a moderate increase of interstitial fluid pressure was found in rat neck tissues using wick catheters (Hargens et al., 1984). Tail-suspended rats also display an increase in intracranial pressure (Maurel et al., 1996), similar to rabbits during HLU or short-term microgravity in a parabolic flight (Florence et al., 1998; Tatebayashi et al., 2003). In mice, we have recently found little change of blood volume and a slight increase in plasma volume using Evans Blue dilution (Popova et al., 2017). In summary, the unchanged blood volume and the increased interstitial fluid volume found here are hard to reconcile with human findings made during space flight, but are in accord with previous reports in hindlimb-unloaded animals.

Relationships between morphological and physiological cardiovascular parameters and body size have been addressed in a

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multitude of studies and have been linked with body mass or length (Calder, 1981; Meijler et al., 2005; Schmidt-Nielsen, 1970). Considering the plethora of size-dependent (heart rate, stroke volume, heart mass, aorta diameter, blood volume, capillary net density, blood flow) and relatively size-independent (blood pressure, hematocrit) cardiovascular parameters (Dawson, 2014; West et al., 1997) affecting, for instance, the filtration–reabsorption balance, we discarded the idea of predicting the slope of an allometric scaling relationship when planning this study, and decided to determine it experimentally. The scaling factors we obtained, summarized in Table 1, are close to previous reports for allometric scaling of erythrocyte count (Kjeld and Ólafsson, 2008) and blood volume (Calder, 1981). We failed to find any data on allometric relationships between interstitial fluid volume and body size, however our estimates of interstitial fluid volume are in accord with existing reports on these parameters in mice, rats and rabbits (Boswell et al., 2014; Courtice and Gunton, 1949; Dreyer and Ray, 1911; Riches et al., 1973).

In order to investigate the dimensional scaling of cardiovascular reactions to hindlimb unloading, we had to obtain data from organisms of different body size, hence the use of three distinct species, which can be listed among the limitations of the study. Unfortunately, mice do not come in three different sizes conveniently spaced by an order of magnitude, which is why we had to adhere to a more realistic choice of laboratory rodents, *Mus musculus* and *Rattus norvegicus*, and a lagomorph, *Oryctolagus cuniculus*, sharing many common features in their body plan and physiology. The second limitation of our study is related to the first; namely, that our data cover only two orders of magnitude. In order to cover a larger range of body sizes, we considered but decided against the use of larger rodents, for instance the beaver or the capybara, primarily because these two species differ a lot in appearance and physiology from the three species used, not the least because of their largely aquatic way of life.

Third, the methodological approaches should be considered, particularly the repeated blood sampling from the relatively small animals and whether it could affect the results of the study. In order to minimize this possible interference, we managed to minimize the blood volume collected to under 5% of circulating blood volume in mice (not more than 100 µl) over the 7 days of the study and to less than 5% for FITC-dextran and -inulin distribution evaluation. The relative amount of blood collected from rats and rabbits was much smaller or negligible. In the case of mice, the data presented here are in good accord with our previous findings in the same species, where we used terminal blood draw. Thus, there is no evidence that the repeated blood sampling in mice seriously affected the results. Considering the pharmacokinetic approach to blood and interstitial fluid volume estimation, we argue that despite higher demands for total blood volume, it is greatly advantageous versus the commonly used single-point dilution measurement. When a single point measurement is employed, the results are very sensitive to the accuracy of blood collection time, because the time points commonly selected are within minutes of probe injection, when the blood concentrations of the probe decrease rapidly (see Fig. 1B) and even a minor time error would result in a large error of distribution volume estimate. In contrast, the V_{ss} derived from a multitude of points on the pharmacokinetic curve is a much more stable estimate.

Table 1. Parameters Y_0 and N for allometric dependency of red blood cell (RBC) count, and blood and interstitial fluid volumes in attached (control) and hindlimb-unloaded (HLU) animals

Parameter	Group	Y_0	N	R^2
RBC count	Control	10.73±0.49	−0.054±0.008	0.6168
	HLU	8.66±0.40		0.4357
Blood volume	Control	0.125±0.015	0.917±0.020	0.9196
	HLU	0.137±0.016		0.8700
Interstitial fluid volume	Control	1.02±0.09	0.648±0.016	0.8990
	HLU		0.708±0.017	0.9668

Y_0, coefficient; N, power.

The loss of RBC mass during a space flight is thought to be an adaptive consequence of plasma volume reduction, enabling maintenance of optimal blood viscosity. In the absence of blood volume changes, other mechanisms must be responsible for the uniform reduction of RBC count in hindlimb-unloaded quadruped animals of different sizes. Although this study was not aimed at revealing the possible mechanisms for RBC loss, a number of possibilities are indicated. Hindlimb unloading induces changes of regional blood flow, particularly to the femur with its relatively limited blood supply (Stabley et al., 2013), which could result in diminished erythropoiesis (Iversen et al., 1992). A putative central venous pressure increase in hindlimb-unloaded animals might diminish erythropoiesis (Montero et al., 2016), along with central mechanisms (Dygai and Skurikhin, 2011). Finally, hematopoietic stem cells seem to be inhibited by clinorotation (Plett et al., 2004). As for the interstitial fluid volume expansion observed in hindlimb-unloaded animals, this seems to reflect the shift of capillary balance towards filtration, at least in the upper body due to additional hydrostatic pressure. This effect, as could be expected, was proportional to body size.

In summary, our results indicate that at least some of the reactions observed under simulated microgravity conditions are relatively size independent. Further experimentation is needed to understand the underlying mechanisms. Despite the fact that our findings, made using small quadruped mammals, cannot be directly extrapolated to humans, it is important to underscore that possibly many mechanisms with different dependency on body size underlie cardiovascular adaptation to weightlessness in humans. Further studies with mice, with a negligible hydrostatic pressure gradient, might help to elucidate the size-independent mechanisms of cardiovascular adaptation to microgravity in humans.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: A.A.A.-A.; Methodology: A.A.A.-A., A.S.P.; Formal analysis: A.A.A.-A.; Investigation: A.S.P., E.A.L.; Resources: A.A.A.-A., O.L.V.; Data curation: A.S.P., E.A.L.; Writing - original draft: A.A.A.-A.; Writing - review & editing: A.A.A.-A., A.S.P., E.A.L., O.L.V.; Supervision: O.L.V.; Project administration: A.A.A.-A.; Funding acquisition: A.A.A.-A., O.L.V.

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