

**FLUID SHIFT VS. BODY SIZE:  
CHANGES OF HEMATOLOGICAL PARAMETERS AND BODY FLUID  
VOLUMES IN HINDLIMB-UNLOADED MICE, RATS AND RABBITS**

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## SUMMARY STATEMENT

Allometric investigation of hematological parameters and body fluids volumes change during simulated microgravity reveals that RBC decrease is size independent, while of ECF volume increase is proportional to body size

## ABSTRACT

Cardiovascular system is adapted to gravity, and reactions to its vanishing in space are presumably dependent on body size. Dependency of hematological parameters and body fluids reaction to simulated microgravity have never been studied as an allometric function before. Thus we estimated RBC, blood and extracellular fluid volumes in hindlimb-unloaded (HLU) or control (ATT) mice, rats and rabbits.

RBC decrease was found to be size-independent, and the allometric dependency for red blood loss in HLU and ATT animals shared a common power ( $-0.054 \pm 0.008$ ) but different  $Y_0$  ( $8.66 \pm 0.40$  and  $10.73 \pm 0.49$  correspondingly,  $p < 0.05$ ). Blood volume in HLU animals was unchanged compared to controls disregarding the body size. The allometric dependencies for interstitial fluid volume in HLU and ATT mice shared  $Y_0$  ( $1.02 \pm 0.09$ ) but had different powers  $N$  ( $0.708 \pm 0.017$  and  $0.648 \pm 0.016$  correspondingly,  $p < 0.05$ ), indicating that 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 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 with 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), 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 spaceflight, 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 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., 2006; Vasilyeva and Bogomolov, 1991), but after landing on other planets both might be limited and thus disadaptation of cardiovascular system might restrict the ability of cosmonauts and/or 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), heart rate (Fuller et al., 2003) and baroreflex sensitivity (Waki et al., 2005) have all been reported after space flights of various duration. Recently, we have extended observations of cardiovascular disadaptation in rodents to space-flown mice using implantable telemetry in the 30-day Bion-M1 biosatellite spaceflight and during the post-flight recovery (Andreev-Andrievskiy et al., 2017). In summary, ample experimental data support existence of cardiovascular adaptation to spaceflight in small quadruped mammals with minimal, if any, hydrostatic pressure gradient.

The model of hindlimb unloading, originally developed by Novikov and Iliyne (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), alterations of blood pressure and/or heart rate (Powers, 2004; Tarasova et al., 2001; Tsvirkun et al., 2012; Zhang et al., 2008), along with other signs of cardiovascular adaptations (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, increase of hydrostatic pressure in the neck tissues (Hargens et al., 1984) and intracranial pressure was reported in the 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 time microgravity in a parabolic flight (Florence et al., 1998). Despite variability of the results with the species used, unloading duration and other experimental parameters, hindlimb unloading, or «suspension» model, proved to be fruitful over the years and still remains virtually the only suitable model for on-ground microgravity effects investigation in animals.

It is well known that “sensitivity” to gravity depends on body size. Nonlinear increase in bone mass in order to support the increasing body weight that limits the size of terrestrial animals (Alexander, 1985) is a nice illustration to this principal. Contrarily, mice can survive roughly tenfold higher acceleration than humans (Chae, 1975), and, an extension of this relationship clearly seen in routine laboratory practice, cells are perfectly viable after centrifugation at hundreds of g’s. When applied to the other side of gravity continuum, microgravity, smaller animals can be expected to be less responsive to vanishing of gravity during a spaceflight or on-ground modeling. This notion complements well with the concept of fluid shifts. Apparently, the larger the hydrostatic pressure, the greater the effects of its elimination should be, and vice versa. Though the fluid shifts have been studied in a series of brilliant studies by Hargens and colleagues using several larger species (Hargens et al., 1987; Lillywhite et al., 1996), it has never been systematically analyzed as a function of body size on the other end of the axis, in smaller mammals. Due to minute body size, hydrostatic pressure gradient in mice cannot exceed  $\approx 2$  mmHg while in the normal posture and  $\approx 5$  mmHg when a mouse rears, thus it could be expected that the changes of body fluids volumes and hematological parameters would be less pronounced in this species compared to larger animals.

Our study was aimed to investigate 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 volumes and hematological parameters in hindlimb unloaded mice, rats and rabbits. We found that in the body size range studied, only 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 fluid shift alone cannot explain the observed changes of body fluid volumes 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 Federal State Budgetary Institution of Science "Scientific Center for Biomedical Technologies of the Federal Medical and Biological Agency" and rabbits from regional rabbit breeding center LLC "KrolInfo". Animals arrived at least two 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<sup>2</sup> for mice, 800 cm<sup>2</sup> for rats) with wood chips bedding (JRS, 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<sup>2</sup>, height – 120 cm), with wooden slatted decking. Standard rodent (Assortiment-Agro, Russia) or rabbit (KrolInfo, Russia) chow and deionized or tap water were provided, correspondingly, to mice/rats and rabbits *ad libitum*. During the hindlimb suspension animals were housed individually. All the 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%, light cycle was maintained at 12.

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

## Experimental design

Two experiments were performed. In the first experiment, hematological parameters were monitored longitudinally in the same animals for the 7 days of hindlimb suspension. In Experiment 2, blood and interstitial fluid volumes were measured on day 3 of unloading compared to 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 automated hemocytometer.

### *Blood and interstitial fluid volumes in hindlimb-unloaded animals*

Blood volume and interstitial fluid volumes were estimated, correspondingly, from FITC-conjugated dextran and inulin volumes of distribution. To calculate volume of distribution, 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 (ATT); 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 three weeks of recovery between the experiments.

## **Hindlimb unloading**

Hindlimb unloading of mice and rats was performed as described by Morey-Holton (Morey-Holton and Globus, 2002) with the modification of Ferreira et al. (Ferreira et al., 2011) used for mice. Briefly, in mice a stainless steel ring was implanted between 3<sup>d</sup> and 4<sup>th</sup> tail vertebra under general and local anesthesia and simultaneously with jugular catheters implantation (described below). In rats, a stainless steel hook was attached with Omniplast adhesive tape (Hartmann, Germany) at the base of the tail after the catheters implantation. Once the animals recovered from anesthesia, they were placed individually into the plastic suspension cages with wire mesh floor (floor area – 600 cm<sup>2</sup>). Next 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 two days. On the third day HLU animals were hindlimb unloaded for 7 days at approximately 30 degrees between the body axis and the cage floor plane, while the ATT 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 1.2x1.1x1.1 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 one day. The next day they were attached to the suspension apparatus that allowed free movement of the animals across the cage. After these two days of adaptation rabbits were either unloaded in HLU experiments or left attached in ATT runs.

## **Blood collection and handling**

In mice and rats blood was collected using jugular catheters in both experiments. Jugular catheters were implanted two days prior to unloading. Briefly, animals were anaesthetized with a combination of zolazepam and tiletamin (15-20 mg/kg each) supplemented with xylazine (5 mg/kg) administered

intraperitoneally. The right jugular vein was accessed through 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 to 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 none weigh loss. The catheters were filled with heparinized (100 U/ml) sterile saline and flushed daily. Blood samples of  $\approx 12$  and 50-60  $\mu$ l per time point were obtained from mice and rats correspondingly using 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, Germany) were implanted for blood collection in experiment 2 using a standard technique. Blood samples were 50-100  $\mu$ l in both experiments.

All the 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 automated hemocytometer 1280vet (Dixon, Russia) and control samples (Streck, USA) for quality control.

### **Blood and interstitial fluid measurement**

Blood and interstitial fluid volumes were estimated from the volume of distribution of FITC-labelled dextran (MW=150 kDa) and inulin (MW=4 kDa), correspondingly. Both probes were purchased from TdB Consultancy AB (Sweden) and purified from unconjugated FITC by dialysis with 1 kDa membrane

vial (Orange Scientific, Belgium) against 1 L water for 24 h protected from light. FITC-dextran, due to large molecular weight, is not subject to extravasation, unlike FITC-inulin, that 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 volumes.

FITC-dextran was injected at a dose of 2 mg/kg to mice and 1 mg/kg to rats and rabbits correspondingly. 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 mg/kg, correspondingly. The differences in doses used for these three species were governed by the need to reduce blood sample volume, and the consequent requirement for greater dilution of the smaller blood samples.

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, 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 M HEPES 9:1 v:v and frozen at -18...-22°C protected from light till subsequent analysis.

The fluorescence of these samples was quantified using Anthos Zenyth 3100 (Biochrom, 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 concentrations.

## Data analysis

Pharmacokinetic data were analyzed using WinNonLin (v.7.0, Certara, USA) software and one-compartmental model for FITC-dextran (Eqn. 1) and two-compartmental model (Eqn. 2) for FITC-inulin correspondingly; among the reported values of distribution volume,  $V_{ss}$  was used.

$$C = A \cdot e^{-\alpha \cdot t} \quad (\text{Eqn. 1})$$

$$C = A \cdot e^{-\alpha \cdot t} + B \cdot e^{-\beta \cdot t} \quad (\text{Eqn. 2})$$

In order to obtain the dependencies of hematological parameters and body fluids volumes on body size they were plotted against body mass (M) in the corresponding animal. The parameters of allometric equations (Eqn. 3) were calculated using Prism (v.6.0, GraphPad, USA). The hypothesis that the equations for ATT and HLU animals have different quotients  $Y_0$  and N was tested using nested F-test with the same software.

$$Y = Y_0 \cdot M^N \quad (\text{Eqn. 3})$$

The body mass corrected values were analyzed with two-way ANOVA followed with Sidak's post-test using Prism (v.6.0, GraphPad, USA).

The differences were considered significant at  $p < 0.05$ . Unless indicated otherwise, the data is presented as mean  $\pm$  standard error of mean.

## RESULTS

### Hematological parameters during hindlimb unloading

As presented in figure 1, erythrocytes count was, expectedly, the highest in mice and the lowest in rabbits, while RBC in rats was intermediate. In all the three species, RBC decreased during hindlimb unloading; the first significant decrease in mice (by  $7.6 \pm 3.6\%$  compared to baseline) was observed as soon as 6 h after unloading, while in rats RBC was decreased compared to baseline values starting from the third, and in rabbits – from the second day of unloading. Generally, RBC decrease progressed slowly over the first three 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 the unloading. Immediately after reloading, a moderate rise in RBC was observed in all three species.

As shown in figure 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), dependencies of red blood cells counts on body mass during attachment and hindlimb unloading were characterized by a shared power of  $-0.054\pm0.008$  ( $F(1,44)=1.54$ ,  $p=0.2208$ ) rather than different powers ( $-0.063\pm0.010$  and  $-0.044\pm0.011$  for ATT and HLU correspondingly). The  $Y_0$  coefficients were however markedly different in ATT ( $10.73\pm0.49$ ) and HLU ( $8.66\pm0.40$ ) states ( $F(1,44)=12.48$ ,  $p=0.0010$ ). Thus, as indicated by a shared slope of RBC dependency on body weight, the hindlimb unloading induced RBC decrease was relatively independent of the body size. The baseline differences in RBC count are compensated by the proportional difference in mean cell volume, thus the hematocrit is close in the three species studied, and similarly to RBC, decreased uniformly with hindlimb unloading (data not shown).

### **Blood volume during hindlimb unloading**

Blood volume was estimated from the volume of distribution of high molecular weight FITC-dextran. When corrected for body mass (fig. 3C), blood volume varied with the species ( $F(2, 38)=19.22$ ,  $p<0.0001$ ) but was similar in hindlimb unloaded and attached animals ( $F(1, 38)=1.37$ ,  $p=0.2484$ ). The allometric functions for blood volume in hindlimb unloaded and attached animals (fig. 3D) were indistinguishable with  $Y_0=0.131\pm0.015$  and the power  $N$  close to 1 and equal to  $0.917\pm0.020$  for both equations, 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  $N$  correspondingly). Thus, there were no differences in blood volume between the hindlimb unloaded and the attached animals disregarding the size of the animal.

## Interstitial volume during hindlimb unloading

Interstitial volume was estimated from FITC-inulin volume of distribution. 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 and edema – in some of the hindlimb-unloaded rabbits, unlike rats or mice. The allometric dependencies of interstitial fluid volume in HLU and ATT animals (fig. 3F) had a shared  $Y_0=1.02\pm0.09$  ( $F(1,37)=1.21$ ,  $p=0.2795$ ) but different powers  $N$ :  $0.632\pm0.030$  for ATT and  $0.720\pm0.014$  for HLU individuals ( $F(1,37)=6.75$ ,  $p=0.0134$ ). The greater power of allometric equation for HLU as compared to ATT animals indicates that the interstitial fluid increase is more pronounced in larger animals.

## DISCUSSION

In this study we addressed the question if changes of hematological parameters and body fluids volumes during simulated microgravity, as indicators of fluid shift, are dependent on the size of the organism. To this aim we investigated reactions of red blood parameters, blood and interstitial fluid volume in hindlimb unloaded mice, rats and rabbits. Using this data we have calculated the allometric equations for the unloaded and attached animals and found out that RBC decrease was relatively independent of body size, blood volume does not change during the 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.

Though frequently applied to diverse physiological systems, scaling approach has been seldom 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 volumes, or other fluid shift related measures in separate species, primarily rats and, less frequently, mice or rabbits.

Reduction of red blood volume is a long established consequence of spaceflight in humans (Leach and Johnson, 1984; Tavassoli, 1982). It has been also 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 be, however, compromised by factors unrelated to microgravity. Unlike real space flight, in the on ground simulation studies with rats RBC and hematocrit are reported to decrease (Dunn et al., 1985; Nezami et al., 2016) or remain unaltered (Chew and Segal, 1997; Ryou, 2012; Saunders et al., 2002). 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 observe in three quadruped species is in accord with some, but not all of the previous findings. We cannot offer a reasonable speculation to explain this apparent incoherence between results from different labs, except that age differences might be implicated, as we prefer to use more mature animals rather than 2-3 months old “teen” rodents. In our hands, RBC decrease is 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.

Decrease of blood and interstitial fluid volumes is another renowned reaction of a 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 was found in a 7-day Cosmos spaceflight (Gazenko and Ilyin, 1987). As for the smaller species, rats, far less pronounced changes were reported using radio-labeled probes during the on-ground simulation (Deever et al., 2002; Somody et al., 1998). Enhanced plasma filtration was found in tail-suspended rats using blood density difference between the arterial and venous blood (Medvedev et al., 1998) and a moderate increase of interstitial fluid pressure was found in the rat neck tissues using wick catheters (Hargens et al., 1984). Tail suspended rats do also display an increase in intracranial pressure (Maurel et al., 1996), similarly 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 are hard to reconcile with human findings made during spaceflight, 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 multitude of studies and have been linked with body mass or length (Calder, 1981; Meijler et al., 2005; Schmidt-Nielsen, 1970). Considering a 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 erythrocytes 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 the existing reports on

these parameters in mice, rats and rabbits (Boswell et al., 2014; Courtice and Gunton, 1949; Dreyer et al., 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 with different body size, thus 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, that 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 one, namely that our data cover only two orders of magnitude. In order to cover a larger range of body sizes, we have considered and decided against 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 due to largely aquatic way of life.

Thirdly, the methodological approaches should be considered, particularly the repeated blood sampling from the relatively small animals and if 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  $\mu$ l) over the 7 days of the study and the same 5% for dextran and inulin distribution evaluation. The relative amount of blood collected from rats and rabbits was much smaller or negligible. In 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 has seriously affected the results. Considering the pharmacokinetic approach to blood and interstitial volumes estimation, we argue that despite higher demands for total blood volume, it is greatly advantageous to the commonly used single point dilution measurement. In case 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 after the 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. Unlike that, the  $V_{ss}$  derived from a multitude of points on the pharmacokinetic curve is a much more stable estimate.

The loss of red cell mass during a spaceflight is thought to be an adaptive consequence of plasma volume reduction enabling maintenance of optimal blood viscosity. Apparently, in absence of blood volume changes other mechanisms must be responsible for the uniform reduction of RBC counts in hindlimb unloaded quadruped animals of different sizes. Despite this study was not aimed to reveal these possible mechanisms or red cells loss, several possibilities might be 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, it seems to reflect the shift of capillary balance towards filtration, at least in the upper body due to an 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 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 negligible hydrostatic pressure gradient, might help to elucidate the size-independent mechanisms of cardiovascular adaptation to microgravity in humans.

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## **AUTHORS CONTRIBUTIONS**

AP and EL have performed the experiments; AAA designed the study, analyzed the data and drafted the manuscript; OV provided funding and scientific oversight of the study; all the authors have revised the draft and approved the final manuscript.

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## **CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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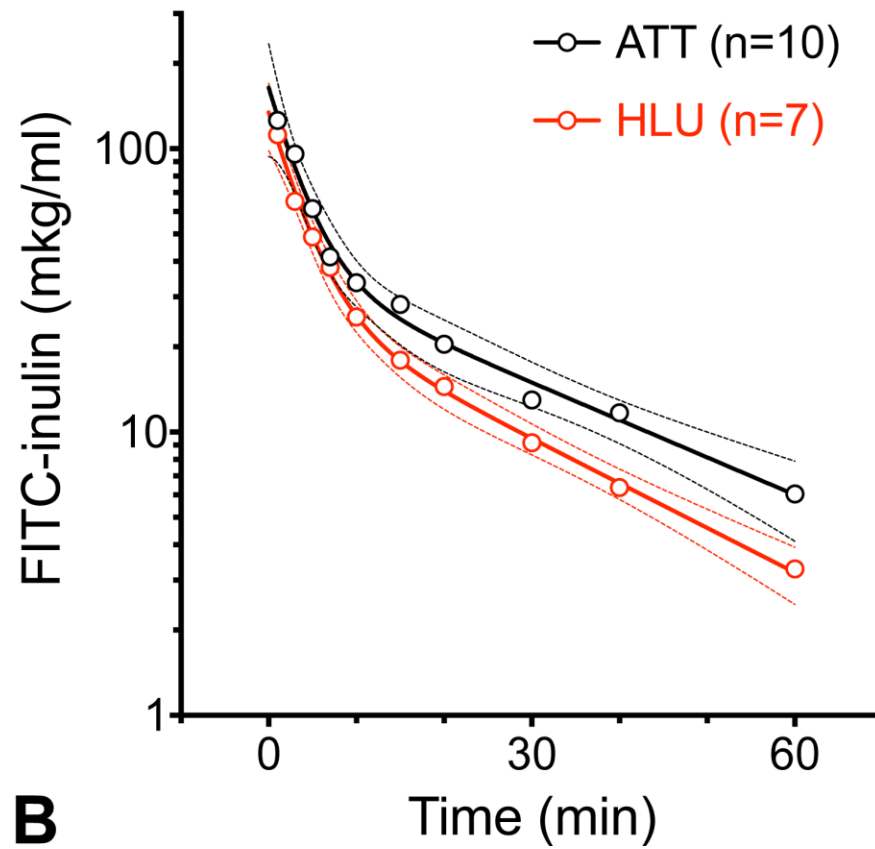
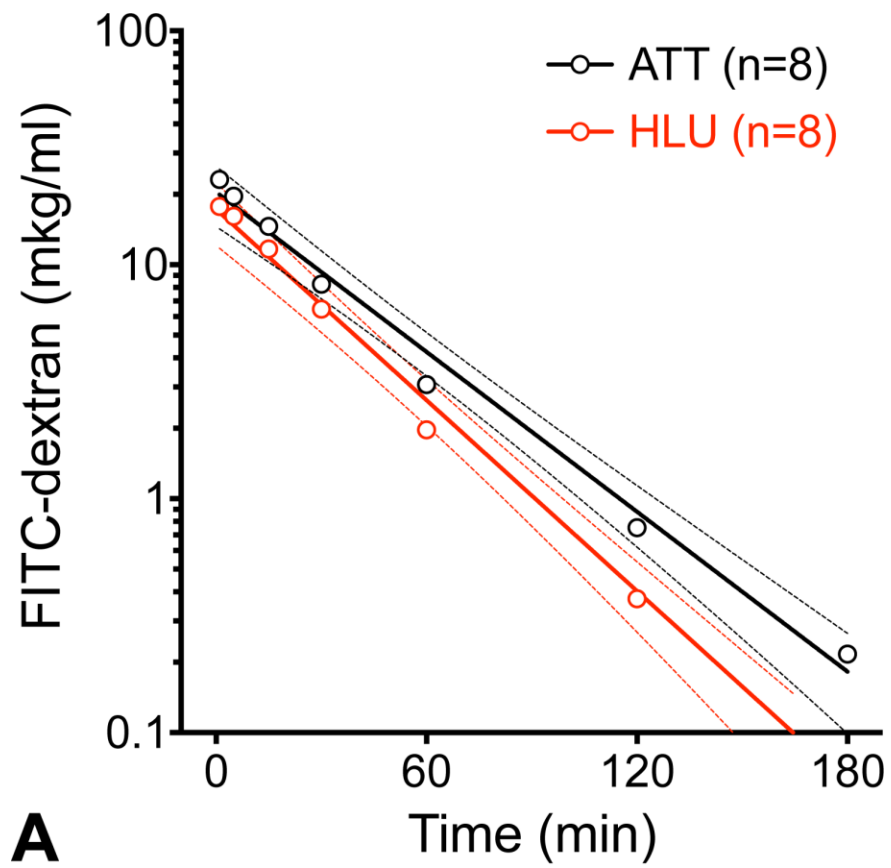
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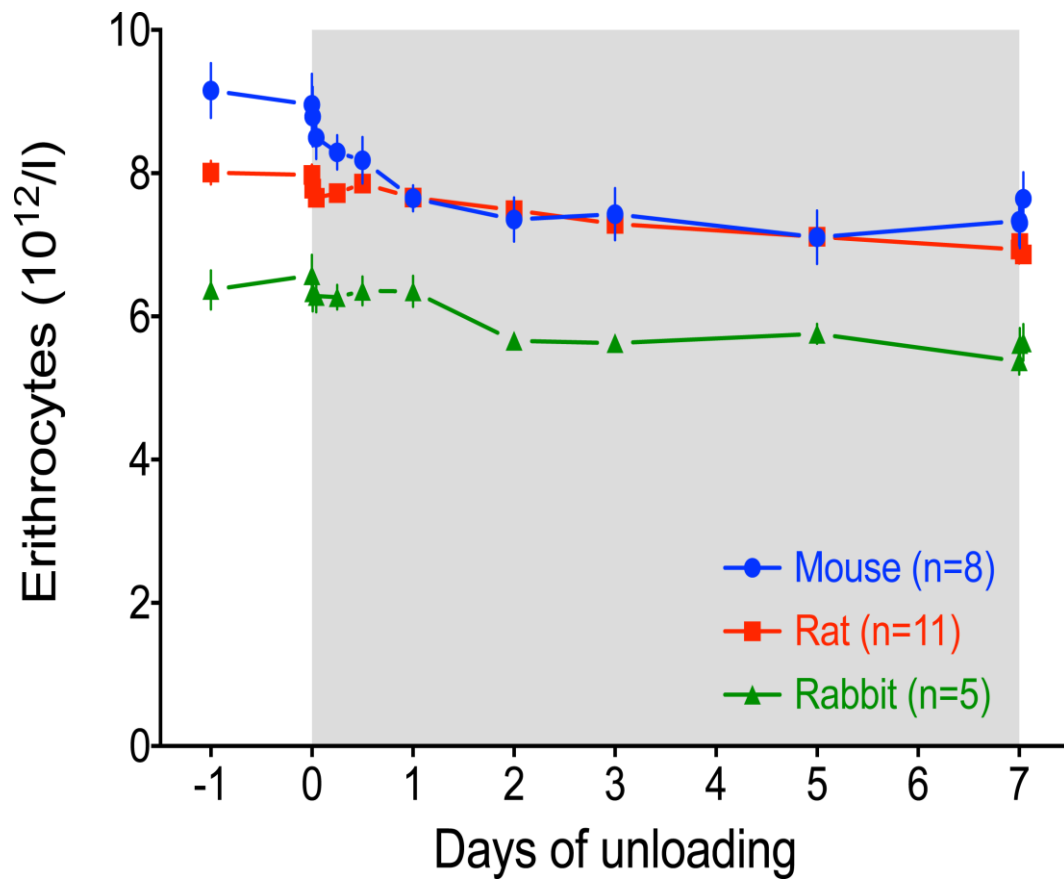
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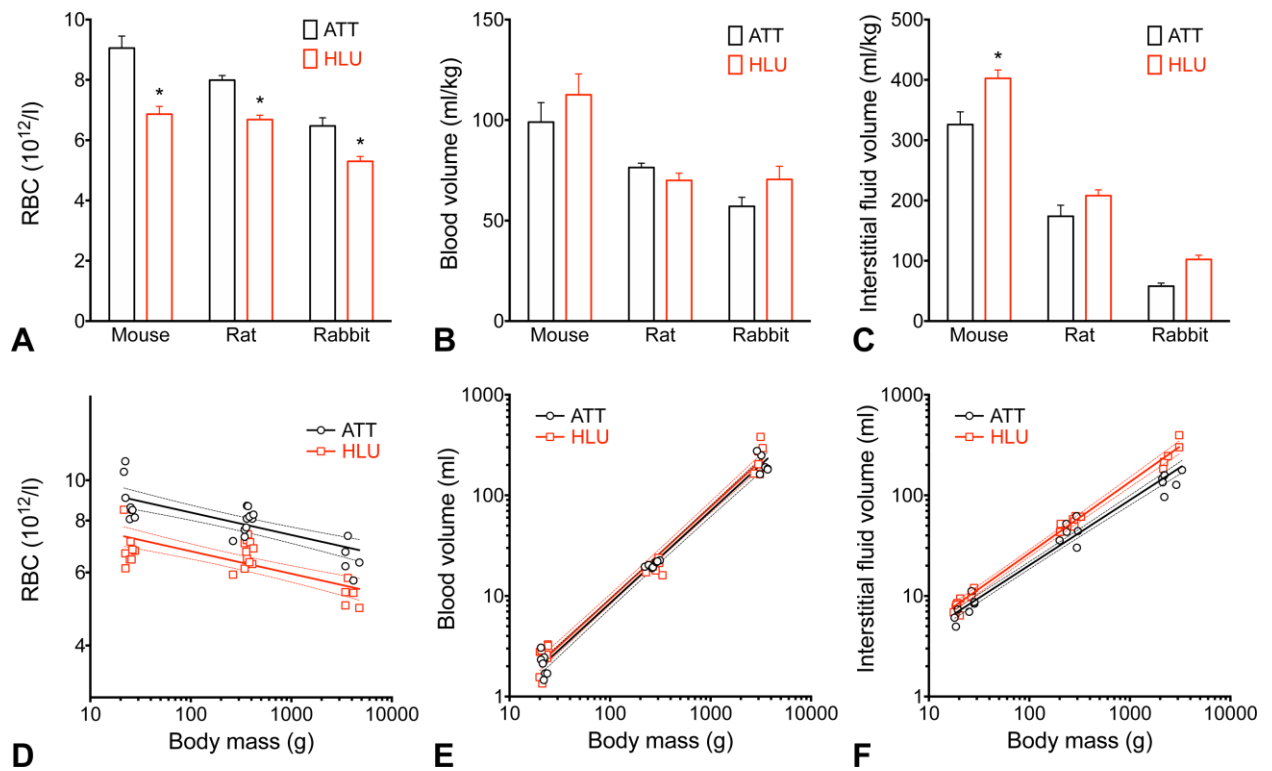
## Figures



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



**Figure 2.** Erythrocytes count ( $m \pm sem$ ) in mice, rats and rabbits during 7 days of hindlimb unloading (highlighted with grey shading). The RBC followed a similar dynamics in all the three species, gradually decreasing over the first three days of unloading and plateauing by day 7.



**Figure 3.** Red blood cells count in hindlimb unloaded or attached mice, rats and rabbits (A) and the log-log analysis of RBC dependency on body mass (B); blood volume corrected for body weight (C) and plotted as a function of body mass (D); corrected for body mass interstitial fluid volume (E) and its dependency on body mass (F). Data is presented as mean  $\pm$  sem in A, C and E. In panels B, D, and F dots represent experimental data, solid lines – regression curves, dashed lines – 95% confidence intervals. Statistics: \* –  $p < 0.05$ , Sidak's test.

**Table 1.** Parameters  $Y_0$  and N for allometric dependencies of RBC, blood and interstitial fluid volumes in ATT and HLU animals.

Parameter	Group	$Y_0$	N	$R^2$
<i>RBC</i>	<i>ATT</i>	10.73±0.49	-0.054±0.008	0.6168
	<i>HLU</i>	8.66±0.40		0.4357
<i>Blood volume</i>	<i>ATT</i>	0.125±0.015	0.917±0.020	0.9196
	<i>HLU</i>	0.137±0.016		0.8700
<i>Interstitial fluid volume</i>	<i>ATT</i>	1.02±0.09	0.648±0.016	0.8990
	<i>HLU</i>		0.708±0.017	0.9668