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The role of impact loading on artery adaptations and the effect of muscle unloading on local blood supply and exercise tolerance

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Hierdurch erkläre ich, dass ich die Leitlinien guter wissenschaftlicher Praxis der Deutschen Sporthochschule Köln eingehalten habe.

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Tobias Weber

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Tobias Weber

„Datt watt esch heij jeschriwwen hann wittmen esch minnger Mamm unn mingem Papp!
Wenn et eisch net juuv wiier dat heij alles net miejellisch jewääst!“

Contents

1 Chapter One – Abstract

Background: Evidence suggests that muscle disuse and exercise training have direct vascular (de-) conditioning effects that can modify cardiovascular risk. The underlying mechanisms, however, are to date not entirely understood. It is clear that particularly arteries adapt to changes of mechanical stress that may predominantly act on endothelial cells (ECs) or vascular smooth muscle cells (VSMCs). Nonetheless, not all potential sources for mechanical stress have been considered yet as previous research basically investigated the conditioning effects of intrinsic hemodynamic forces. Given the gravitational environment on earth, the present thesis sought therefore to investigate the impact of gravity-induced impact loading, provoking mass accelerations, on arterial adaptations in conditions associated with exercise training and muscle disuse. Moreover, the relationship between local arterial blood flow, muscle perfusion and dynamic exercise tolerance after prolonged local muscle disuse constitutes a major topic of the present thesis, as to date no study has ever elaborated on this.

Methods: Two clinical interventional studies have been carried out. Within the scope of the HEP-study, 11 healthy male subjects wore a new orthotic device (HEPHAISTOS) for 56 days to unilaterally unload the calf muscles without changing the impact of gravitational loading. The EVE-study has been conducted to study the effects of whole body vibrations (WBV) if combined with conventional resistive exercise. 26 healthy male subjects were recruited and assigned to either a resistive exercise group or a resistive vibration exercise group that performed a 6 week training intervention. Major endpoint measurements for both studies encompassed structural and functional arterial parameters as measured with sonography. In addition, soleus muscle morphology and tissue oxygenation have been assessed in the HEP study using soleus muscle biopsies and near infrared spectroscopy, respectively.

Results: Muscle unloading with the HEPHAISTOS orthosis led to distinct decreases of superficial femoral artery (SFA) calibers, while wall thickness and endothelial function remained unaffected. Although muscle size and arterial calibers significantly decreased, functional exercise blood flow, tissue oxygenation and exercise tolerance did not change after the intervention. During the EVE-study, SFA calibers increased significantly and carotid artery wall thickness decreased significantly in response to resistive exercise while superposition of WBV did not reveal an additional effect.

Conclusion: Both studies highlight the importance of muscle work and hence the importance of intrinsic hemodynamic forces for arterial adaptations. However, gravity-induced impact loading seems to have a direct conditioning effect on arterial wall thickness and on endothelial function, thereby modulating parameters for cardiovascular risk. In addition, functional muscle perfusion seems to remain unaffected after prolonged muscle disuse and possibly as a consequence of this, dynamic exercise tolerance does not change.

2 Chapter Two – Introduction

The arterial system consists of a complex network of blood vessels and functions as transportation network for many cells, hormones and a multitude of other molecules within the human body. Structural as well as functional arterial properties within this system vary and are largely determined by the anatomical region they supply. For instance, dimension and function of the carotid artery (CA) strongly differs in terms of size and function compared to arterioles in calf muscles. However, the general construction of arteries is always the same, as one can basically differentiate between three main layers: a) the tunica intima, in the following referred to as 'Intima', is the innermost layer of an artery that is in direct contact with the circulating blood, consisting of endothelial cells (ECs); b) the tunica media, in the following referred to as 'Media', is the middle layer which is composed of vascular smooth muscle cells (VSMCs) and elastic tissue and c) the outer tunica adventitia, in the following referred to as 'Adventitia', consisting of mainly collagen fibers to anchor the artery to surrounding tissue (see Figure 1; 59).

Yet, the arterial system does not merely function as a passive apparatus of rigid pipes through which blood flow is merely controlled by heart action. In fact, blood flow is fine regulated by cardiac and by vascular function in order to meet the specific metabolic demands at rest and during physical activity. Particularly peripheral blood flow supplying skeletal muscles is greatly dependent on peripheral vascular tone that in turn is orchestrated by a complex interplay of various neuro-humoral, metabolic and mechanical control mechanisms acting on VSMCs and ECs (52). Both functional as well as structural properties of arteries show the ability to acutely and chronically adjust to different functional demands and varying environmental conditions and certain parameters of arterial structure and function are thought to be directly related to physical fitness and cardiovascular risk (91).

As a matter of fact, mechanical stress acting on the arterial wall is considered as the major signal to induce structural and functional artery adaptations. Numerous in- and ex-vivo studies revealed a direct conditioning effect of stresses like 'shear', 'pressure' and pressure-related 'stretch' upon ECs and VSMCs (15; 40; 55; 108). Thus, arterial wall *shear stress* would be triggered by the action of blood flow, as a consequence of blood cells applying dragging forces on ECs. On the other hand, circumferential *stretch* would be generated by pulsatile blood pressure, stretching the artery and thus the connected cells in the arterial wall during

every cardiac cycle, while intravascular *compression* would be induced as a result of systemic blood pressure (22; 100).

Since blood flow as well as blood pressure are directly affected by muscle work (52), conditions associated with physical activity or physical inactivity have a direct conditioning or deconditioning effect on blood vessels, respectively, which is particularly exerted on arteries (93). However, considering our gravitational environment on the surface of earth, there must be another potential source for mechanical stress acting on the arterial wall that acts independently from blood flow and pulsatile blood pressure. In this context, given the nature of habitual activities, it seems to be reasonable to examine gravity-induced impact loading more closely as a potential source for mechanical signaling inducing vascular adaptations. Figure 1 depicts all forces acting on arteries that may occur, given habitual locomotion activities on earth.

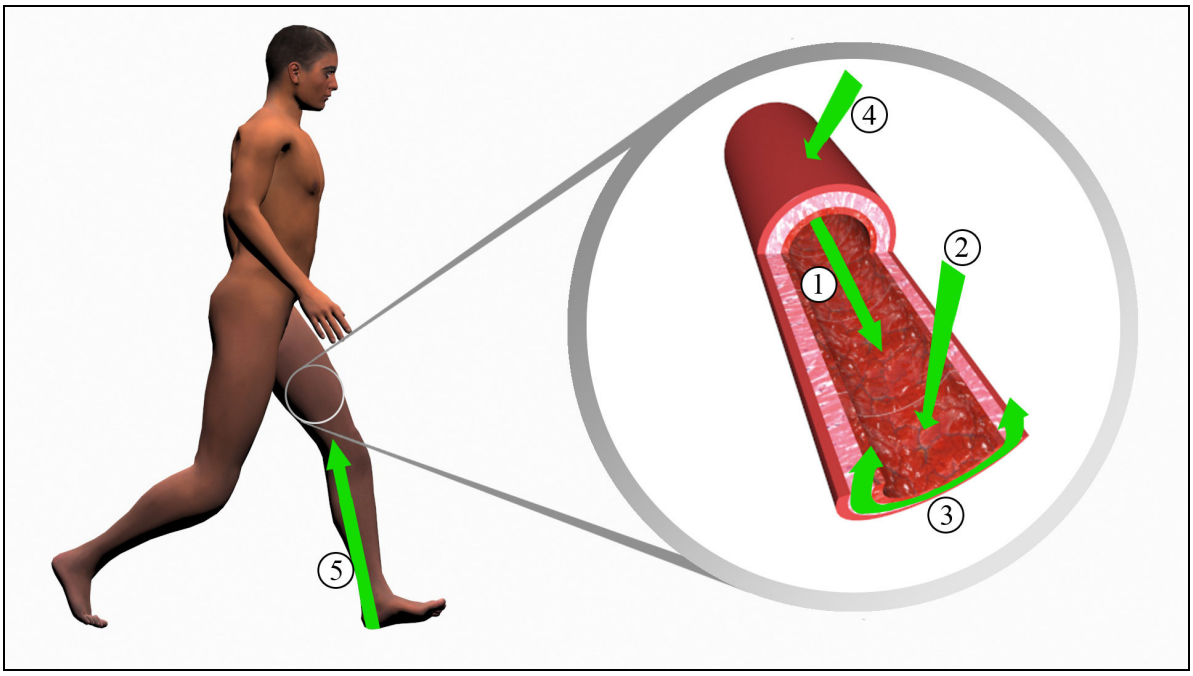

Figure 1. Sources for mechanical stress acting on the arterial wall. The arterial wall consists of the three layers Intima, Media and Adeventitia (from the inside to the outside). Green arrows labeled with numbers 1-3 indicate intrinsic hemodynamic forces that are induced by the action of blood: (1) *shear stress*, dependent on blood flow velocity and arterial caliber (2) *compression*, dependent on blood pressure (3) pulsatile stretch (*strain*), induced by the pulse wave. Arrow (4) indicates extravascular forces, induced through e.g. muscle contractions. Arrow (5) indicates ground reaction forces that occur during each gait cycle, leading to gravity-induced impact loading and thereby to mass accelerations of human tissues. Figure modified from Dancu et al. (22).

Gravity-induced impact loading occurs during any given habitual activity as a result of body-ground interactions and the resulting ground reaction forces are associated with vertical tibia accelerations of up to 10 g during running (53). Given the amount of 4000 to 18000 steps per day in healthy individuals (101) there are a considerable number of habitual accelerations being generated every day, generating mechanical stress in the human systems, in particular the systems of the lower extremities. However, thus far, there have been no studies addressing the specific effect of gravity-induced impact loading on arterial adaptations. Consequently, the investigation of the specific role of gravity-induced impact loading and its effect as mechanical trigger upon structural and functional artery adaptations constitutes a central topic of the present thesis that is organized as follows:

Chapter One: Abstract of the thesis

Chapter Two reviews the scientific background of the investigated main parameters concerning vascular adaptation and muscle perfusion. In addition, methodological aspects of the two conducted studies and the purpose of this thesis are presented.

Chapter Three comprises three scientific papers with the central topic of structural and functional vascular adaptations and one scientific paper addressing methodological issues of the ambulatory HEPHAISTOS study.

Chapter Four summarizes the major research findings and final conclusions are drawn.

Chapter Five contains the original papers, the curriculum vitae of the author and an abstract in German language.

2.1 Structural artery adaptations

Two major parameters of arterial structure are the vessel caliber and the vessel wall thickness. Given the distinct ultrasound reflections of Intima and Media, arterial diameters and wall thickness can be measured very accurately in vivo using high frequency B-mode sonography (67). Particularly large and superficial conduit arteries like the carotid artery or the superficial femoral artery (SFA) constitute an ideal measurement site for ultrasound measurements (Fig. 1).

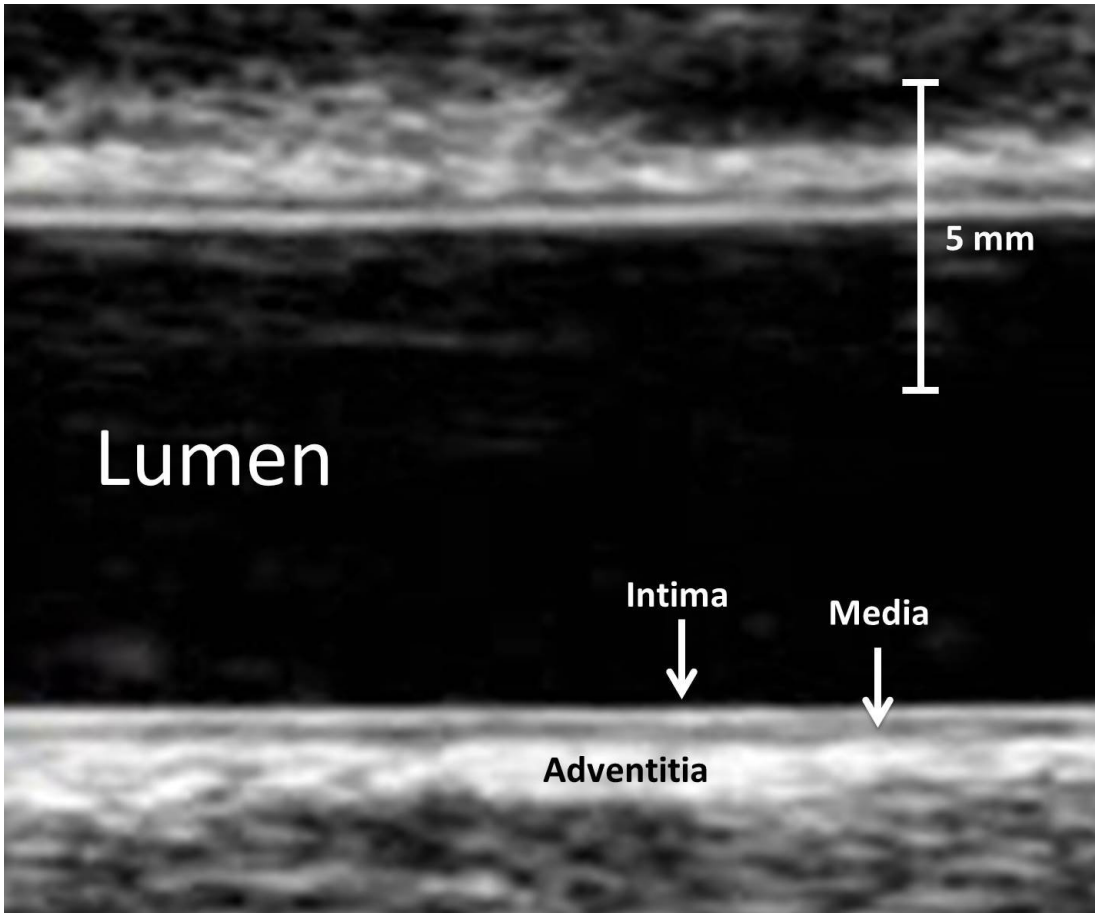

Figure 2. Ultrasound B-mode image of the CA. The image shows the longitudinal section of the CA with the distinct wall reflections of Intima and Media layers. The far wall (bottom) of the vessel is taken for Intima Media Thickness analyses.

Structural artery parameters provide a meaningful surrogate measure for the quality of the cardiovascular system, as an example, the Intima Media thickness (IMT) of the CA is commonly being used to predict cardiovascular risk and to monitor (pre-) clinical cardiovascular diseases (CVD) (27; 63). Previous studies showed that physical activity and inactivity have a direct conditioning effect upon IMT and arterial calibers, meaning that muscle work and disuse can be directly associated with arterial remodeling and hence with cardiovascular risk (87; 104).

2.1.1 Adaptations of Intima Media Thickness (IMT)

Evidence suggests that IMT adaptations are rather driven through alterations of blood pressure than through alterations of *shear stress* (87). Accordingly, chronically elevated blood pressure in hypertensive subjects leads to arterial wall thickening (70) and changes the proliferation and growth characteristics of VSMCs in vitro (106). In contrast to this, cyclic

pressure-related *stretch*, in the following referred to as '*strain*' seems to have a pronounced effect on ECs that is, at least in vitro, greater than the effect of mere pressure-induced *compression*, leading to the expression of both pro- (e.g. ICAM-1) and anti-atherogenic (e.g. eNOS) genes (1; 14). Given these partly controversial findings in vitro, generalized conclusions about the pro- or anti-atherogenic effects of the mechanical signals *compression* and *strain* are difficult to draw and seem to be dependent on variables like duration, frequency and magnitude (55). The general in vivo-finding that IMT decreases in response to exercise (87) and increases as a consequence of muscle disuse (104) should thus also be investigated from another perspective, since local and central blood flow and blood pressure changes in situations related to muscle work and muscle disuse cannot entirely account for the observed IMT adaptations in vivo. It therefore seems plausible to likewise investigate the effects of gravity-induced impact loading on IMT adjustments in vivo, in situations associated with muscle unloading and exercise training.

2.1.2 Arterial caliber adaptations

Although adaptations of arterial wall thickness are linked to arterial caliber adaptations, it seems that the role of mechanical forces for adaptations of arterial size is a bit more illuminated. The size of the arterial lumen adapts to changes of wall *shear stress*, which in turn is predominately dependent on blood flow velocity and on arterial calibers (54; 102). So, for instance, the radial artery caliber does significantly increase in renal disease patients with arteriovenous fistula, where the excessive reduction in peripheral resistance leads to massive increases of blood flow (36). Accordingly, it has been suggested that reductions of arterial wall *shear stress* provoke arterial inward remodeling, leading to smaller arterial calibers (107). The observation that arterial calibers increase in response to exercise training (37; 62; 90) and decrease as a consequence of unloading (9; 10; 105) is thus mainly attributed to increased or decreased wall *shear stress* following muscle work, or muscle disuse, respectively (93). Importantly, these caliber adaptations do not occur when the EC layer is removed (54), indicating that mechanical signals provoking arterial remodeling are initially sensed by ECs and in addition, that intact EC layers are crucially needed for this adaptation process. Regardless of the apparent close relationship between *shear stress* and arterial caliber adjustments, it seems yet reasonable to consider also gravity-induced impact loading as a potential source for mechanical signaling, leading to adaptions of arterial size.

In light of the importance of ECs in relation to structural artery remodeling, the following paragraph addresses functional artery adaptations, of which the so-called flow mediated dilation (FMD) provides a valid surrogate marker for endothelial health and thus for general cardiovascular health (21; 47).

2.2 Functional artery adaptations

In the following, arterial function will refer to (i) flow mediated dilation (FMD), i.e. the endothelium-dependent ability of an artery to dilate and (ii) to blood flow as the blood volume which is flowing through an artery per unit time that is dependent on arterial calibers and flow velocity (26).

2.2.1 Flow mediated dilation (FMD)

It is clear that arterial function in terms of blood flow is related to its structure, as bigger arteries can transport larger blood volumes compared to small arteries. In addition, also for the dilation capacity of an artery it seems that the structural property wall thickness has a pronounced impact (94). However, arterial function in terms of FMD does not solely follow arterial structure and it could be shown that functional adaptations can precede structural adaptations following periods of exercise training or muscle unloading (24; 93; 96). Based on Duplex sonography, FMD tests are nowadays commonly used to investigate the nitric oxide (NO) -dependent dilation capacity of arteries. If applied under strictly standardized conditions, FMD tests allow the evaluation of endothelial driven dilation of superficial conduit arteries (21; 47). Typically, a distinct FMD response is associated with cardiovascular health, whereas an impaired FMD response can be found in cardiovascular disease (CVD) patients (12; 110). In this context, endurance exercise training seems to have a beneficial effect upon FMD in healthy individuals (16; 96) that is even more pronounced in subjects with increased cardiovascular risk (93). Contrary to the clear trend of FMD adaptations related to exercise training, FMD adaptations following disuse appear to be rather unexpected. Previous unloading studies reveal that the FMD response remains unaltered or even increases following muscle disuse (9; 28; 105). This dilation overshoot can be partly explained by the increased *shear stress* that occurs as a consequence of smaller arterial diameters, leading to a stronger dilation stimulus following periods of disuse. If normalized to shear stress, it could be shown that FMD remains unaffected, or only marginally increased, even in spinal cord injured (SCI) individuals (26; 92). These findings disclose that functional artery adaptations to

disuse are not simply the inverse of exercise induced adaptations. Thus far, the underlying mechanisms for the preserved FMD after physical inactivity are not entirely understood. It was suggested that increased smooth muscle sensitivity to NO might contribute to the maintained FMD after prolonged physical inactivity (93) as previous studies found that the dilation magnitude in response to administration of the NO-donor nitroglycerin was more pronounced after muscle unloading (9; 10). However, opposite to the above findings, a more recent study reveals that NO sensitivity does not differ between SCI individuals and able bodied controls (92), questioning the increased NO-sensitivity of VSMCs as a consequence of physical inactivity. Thus, the reason for the unaltered FMD in response to muscle unloading is yet unknown. Nonetheless, it is clear that NO-signaling, also involved in various other adaptation processes of skeletal muscle and vascular systems (85), plays a crucial role in FMD adaptations. Especially the role of the endothelial derived nitric oxide synthase (eNOS) seems to be essential in this context as eNOS mRNA levels and protein expression as well as eNOS activation are triggered by *shear stress* (4; 35; 73; 109). During muscle work, *shear stress* increases as a consequence of increased blood flow. The observed increased FMD response following exercise interventions can thus be mainly attributed to a greater bioavailability of eNOS that allows for a greater *shear stress*-induced expression of NO (38; 109). Notwithstanding the obvious crucial impact of *shear stress* upon FMD adaptations, the effects of gravity-induced impact loading is also worth being considered in this regard. To this day no exercise- or disuse study has ever addressed the specific role of gravity-induced impact loading on endothelial function in terms of FMD.

In light of the above considerations, the specific effects of habitual gravity-induced impact loading on FMD, resting blood flow, IMT and arterial caliber adaptations during prolonged muscle unloading are elaborated in *paper two* of the present thesis, while *paper four* addresses the effect of resistive exercise and superimposed artificial impact loading on structural and functional arterial parameters.

In the following paragraph the adjustment of blood supply to working muscles will be elaborated.

2.2.2 Skeletal muscle blood flow

It is one aim of the present thesis to investigate potential changes of exercise blood flow after prolonged muscle disuse and to assess whether this conceivably correlates with changes of exercise tolerance.

2.2.2.1 From resting blood flow to exercise hyperemia

Given the comparatively low metabolic demand of muscle tissue at rest (111), muscle perfusion is poor and claims only a fraction of the cardiac output. However, right after the onset of muscle work, blood flow and muscle perfusion increase dramatically to a multiple of resting conditions (52; 111). Functional and structural adaptations of the vasculature are therefore particularly relevant in relation to muscle performance. During muscle work blood flow is orchestrated in a complex fashion, given the various central and local cardiovascular adjustments and the multitude of neuro-humoral, mechanical and metabolic factors that are involved (52). A single muscle contraction leads to elevated muscle perfusion, most likely as a consequence of an increased arterial-venous pressure gradient resulting from the so-called 'muscle pump' function (82). This mechanism seems to be of particular importance during upright and rhythmic activities, for instance during walking, in order to allow for sufficient and sustained blood supply right after the onset of muscle activity (82). However, in order to match the metabolic demand during on-going muscle contractions, further adjustments of the cardiovascular system are required that depend on the involved muscle volume, the workload and the duration of muscle work (72; 80). On-going exercise leads to elevated activity of the sympathetic nervous system (64) inducing vasoconstriction in inactive tissue, thereby increasing mean arterial blood pressure (MAP). Sympathetic activity furthermore increases heart rate and cardiac contractility as well as inspiration frequency and –depth, resulting in increased cardiac output and oxygen volume (VO_2) -intake. Simultaneously occurring vasodilation of arteries and arterioles supplying exercising muscles provokes a drop of peripheral resistance and thus a shift of blood volume away from non-exercising muscles and peripheral organs towards exercising muscles that can, depending on the involved muscle mass and workload, account for up to 85% of the cardiac output (52; 68). Consequently, more oxygen will be transported to working muscles, whereas non-exercising muscles and peripheral organs run in 'energy saving-mode' (52). While the sympathetic vasoconstriction is centrally driven through α-adrenergic signaling (60), exercise-induced vasodilation occurs locally in response to various biochemical and mechanical signals. The main aim of all

vasodilation signals is of course to inhibit cross bridge binding between actin and myosin filaments in VSMCs and thus to inhibit vasoconstriction. Importantly, any change in vascular tone is linked to VSMC cytosolic free calcium (Ca^{2+}) concentrations and/or the sensitivity of contractile proteins to Ca^{2+} (41; 52). Following increased cytosolic Ca^{2+} levels, the formation of Ca^{2+}-Calmoduline complex is fostered and the myosin light chain kinase (MLCK) gets activated, which in turn phosphorylates regulatory sites on the MLC. This finally leads to docking of myosin- heads to the actin filaments, which initiates cross-bridge formation and vasoconstriction (66; 71).

As stated in the previous paragraph, flow mediated dilation (FMD) constitutes a major vasodilation mechanism following increased levels of *shear stress* and the FMD response is basically dependent on the magnitude of *shear stress* and the bioavailability of NO (49). The latter is mainly dependent on eNOS concentrations in ECs. Following bouts of muscle work, endothelial *shear stress* is elevated and induces eNOS activation and NO production (4). Nitric oxide in turn acts as a powerful vasodilator that activates the cyclic guanosine monophosphate (cGMP) pathway, mainly evoking decreases of intracellular free calcium concentrations and thereby inducing -relaxation in VSMCs (57).

In contrast to the flow mediated dilation effect of elevated *shear stress* provoke increases of blood pressure (thus increases of wall tension and *strain*) VSMC myogenic regulation towards vasoconstriction (23). In exercising muscle, this blood pressure-related myogenic regulation competes with many other vasodilation mechanisms, however, it is believed that its role during exercise is only secondary (52). Another mechanical stimulus that could be of importance for exercise-induced vasodilation is suggested to be extravascular pressure (see Figure 1) induced by contracting muscles (17). It appears that arterial diameters respond to increases of extravascular pressure greater than 100 mmHg (2) and it seems that this response is dependent on both endothelium and VSMCs (19).

During on-going muscle contractions, prolonged vasodilation is also triggered by various metabolites and other substances that emerge during muscle work and it could be shown that ECs, skeletal muscle cells and erythrocytes may account for potential sources to release vasoactive substances (18). Depending on morphological properties of the working muscles, there are different substances being considered to provoke vasodilation (52). Mainly considered in this context are: adenosine, accumulating in the interstitial space (7), potassium

that is released following every action potential (11), lactate and CO_2 that evoke a pH drop in the interstitial space (58), and ATP that is suggested to be released during exercise from ECs, erythrocytes and sympathetic nerves (13). Moreover, changes of oxygen partial pressure (PO_2), increases of tissue osmolarity, cytokines as well as components in the extracellular matrix seem to be involved in local exercise-induced vasodilation. It is also suggested that many other to date unknown factors might contribute to this complex regulative mechanism (52).

Once the local dilation process is initiated in peripheral arterioles of skeletal muscles, the dilation signal is transmitted upstream to at least three branch orders of arterioles proximal to the initially stimulated arteriole (61). The so-called conducted vasodilation occurs in response to electrical signaling ascending through gap-junctions in ECs, transmitting the dilation signal to the adjacent VSMC layer (3). The mechanism of conducted vasodilation is understandably of great importance to allow for sufficient blood supply in working muscles.

2.2.2.2 Long term adaptations

Given muscle perfusion and exercise tolerance as one major topic of the present thesis, the following paragraph will primarily elaborate on vascular adaptations in relation to exercise tolerance in conditions associated with physical activity and muscle unloading

It is clear that an exercising muscle has a higher demand for oxygen and substrates that are metabolized to produce adenosine triphosphate (ATP), the ultimate power source for muscle work and various other cell activities (34). Thus, the major limiting factor for exercise tolerance is given by the efficiency of working muscles to utilize oxygen and substrates in the process of oxidative phosphorylation to produce ATP (42). The turnover rate in the production of ATP is mainly dependent on oxygen delivery and oxygen utilization (44). The VO_2 that can be taken up and transported to the working muscles depends on structural and functional properties of the cardiovascular and pulmonary systems. Accordingly, heart rate, heart size, stroke volume and hence cardiac output as well as muscle capillarization and pulmonary gas exchange capacities constitute limiting factors for VO_2-uptake (45). Furthermore, the number of erythrocytes and as a result the hemoglobin concentration, determines O_2 binding and transport capacity of circulating blood (33). More recent work suggests even that the deformability of erythrocytes might also play a role in O_2-delivery as more flexible erythrocytes can better travel through smallest capillaries (84). On the other

hand, oxygen extraction and utilization are dependent on the oxidative capacity of muscle cells that is mainly determined by density, structure and function of mitochondria (43). It appears logical that the diffusion distance from erythrocytes through the endothelium into mitochondria of skeletal muscle cells constitutes a major limiting factor in the process of muscle oxygen supply. Thus, oxygen diffusion distance depends on tissue capillarization and fiber size. Muscle tissue is characterized to be highly adaptable to its environmental conditions (83) and it is known that beyond other paradigms, e.g. in pathological conditions, exercise training (39; 95) and muscle unloading (103) have the greatest potential to alter muscle morphology in terms of muscle capillarization and muscle cell size. Therefore, oxygen diffusion distance may vary from exercise trained to physically inactive muscles. The phenomena that muscle cells hypertrophy in response to (resistance-) exercise training and atrophy following muscle disuse has been described extensively in previous research (48; 81). However, it is with respect to the topic of the present thesis of particular importance to investigate the consequences of muscle unloading for exercise tolerance and muscle oxygen supply.

The formation of new capillaries is generally referred to as angiogenesis and it may occur in conditions associated with health (e.g. formation of capillaries in skeletal muscle) or disease (e.g. tumor growth) to better supply tissue with oxygen (65). Angiogenic capillary growth can be triggered by numerous biochemical mediators of which the often mentioned vascular endothelial growth factor (VEGF) and angiopoietins are only two well-established factors (65). Thus, it could be shown that hypoxia and endothelial *shear stress* may act as potent signals to initiate biochemical signaling pathways inducing angiogenesis (29; 32; 65).

Currently, there are two known mechanisms to promote angiogenic neovascularization that have been denominated *sprouting angiogenesis*, as the formation of new capillaries following ECM degradation, EC migration and proliferation to form new capillary tubes and *intussusceptive angiogenesis*, where an existing capillary divides longitudinally to form two capillary vessels (56; 69). Moreover, formation of new skeletal muscle capillaries may also result from vasculogenesis, a process promoted by the release of bone marrow-derived endothelial progenitor cells (EPCs), that has for long time only been ascribed to occur in embryonic neovascularization (30). It is obvious that blood vessel growth in skeletal muscle can distinctly contribute to improvements of exercise tolerance via decreasing diffusion distances from erythrocytes to skeletal muscle mitochondria. On the other hand, the opposite

of neovascularization, namely capillary regression, should lead to an impaired exercise tolerance as oxygen supply to working muscle fibers should be hindered. Nonetheless, while the formation of new capillaries has been extensively studied in relation to exercise training, capillary adaptations in response to muscle unloading have been less investigated. Given hypoxia and mechanical *shear stress* as the two main stimuli that are considered to trigger formation of new blood vessels (29; 32; 65) it appears logical to expect capillary regression in response to muscle unloading, primarily because blood flow, thus *shear stress*, in unloaded muscles is greatly reduced or even completely abolished in distant capillary beds (46). But then again, if capillarization in response to reduced *shear stress* gets less, tissue oxygenation also decreases and hypoxia-induced formation of new capillaries should be triggered. A previous study suggests in this context that angiogenesis occurs in response to tissue hypoxia following ligation of an artery but also that the angiogenic response is greater when bouts of *shear stress* are applied (46). These findings reinforce the important role of *shear stress* for angiogenesis but they suggest also that when muscles are poorly perfused for a longer period, hypoxia-induced angiogenesis might contribute to the development of an equilibrium between capillary regression and neovascularization. Previously conducted studies reveal that capillary regression occurred very quickly following unloading and moreover that capillary regression plateaued after 1-3 weeks of disuse (51). It was also observed that the regression of capillaries was more prone in predominantly slow muscle fibers (79). However, together with the simultaneous occurrence of muscle atrophy, capillary density was observed to remain unchanged or even increased (51), suggesting unaltered or even shorter diffusion distances between capillaries and muscle cell mitochondria after prolonged muscle disuse. Consequently, the arising question should be if local functional muscle perfusion, oxygen supply to working muscles and exercise tolerance are deteriorated after long-term muscle disuse. While evidence suggests that the overall VO_2 capacity is reduced after muscle disuse (20), it is to date not clear whether local vascular adaptions can account for the decreased whole-body utilization of oxygen. It therefore seems to be reasonable to investigate the effects of local muscle unloading on functional muscle perfusion, oxygen delivery and exercise tolerance and the results of this investigation are presented in *paper three* of the present thesis.

2.3 Conducted studies and applied models for exercise training and muscle unloading

All data that are presented in this thesis have been acquired within the scope of two ambulant clinical interventional studies that will be introduced in the following.

2.3.1 The HEPHAISTOS study (HEP-study)

The HEPHAISTOS study (HEP-study)

A novel orthosis has been developed within the frameworks of the present PhD project and the PhD project of *Michel Ducos* in order to locally study the specific effects of gravitational loading upon muscles, vasculature, bones, cartilage and nerves independently from muscle contraction forces. The HEPHAISTOS unloading orthosis allows normal ambulation without crutches and thus retains gravitational loading, while plantar flexor activation and torque production are greatly reduced (Fig.2). The technical principles of HEPHAISTOS-unloading have been patented (patent application number: 102011082700.5) and a detailed biomechanical analysis of the HEPHAISTOS has been addressed in the thesis of Michel Ducos that will be published elsewhere (31). In order to investigate physiological long-term effects of wearing HEPHAISTOS and to compare them with the effects of other established disuse models like bed rest (6) or unilateral limb suspension (ULLS) (8), eleven healthy male volunteers wore the HEPHAISTOS orthosis for 56 days during all habitual activities that required loading of the legs. The ambulant interdisciplinary multicenter HEP-study was carried out from April 2011 to June 2012 (including all baseline and follow-up measurements).

Figure 3. The HEPHAISTOS unloading orthosis

During this time, subjects visited the laboratory at least once per week for measurements and reports. The HEP-study, underlying the first three papers of the present thesis, is registered as a clinical trial at www.clinicaltrials.gov (Identifier: NCT01576081). Details about study design, subject recruitment, reambulation and other methodological aspects have been addressed in *paper one*.

2.3.2 The molecular and functional effects of resistive vibration exercise study (EVE-study)

Whole body vibration exercise (WBV) has been considered as a novel training modality with various potential fields of application that has sparked more and more interest in the past decade (74). Previous studies explored numerous physiological acute and long-term effects of varying vibration stimuli leading to artificial high-frequent impact loading. In doing so, different amplitudes, different vibration frequencies, diverse muscle contraction forms and diverse vibration devices were investigated (74-78; 97-99; 105). The EVE-study, underlying the results of *paper four* of the present thesis, was carried out at the German Aerospace Center

(DLR) in Cologne and at the University Hospital in Cologne in order to gain more physiological knowledge about the effects of resistive vibration exercise. Twenty-six volunteers participated in the exercise study and were assigned to either a vibration group where subjects performed six weeks of resistive exercise with superimposed vibrations or an exercise control group where subjects performed the same training without vibration stimulus. The training regime comprised 2-3 bouts per week while each bout consisted of three squat and three heel raise sets. Additional weights were applied during all exercises using a guided barbell. Throughout the six exercise intervention weeks, vibration frequency was increased and training weights were adjusted to gains of muscle strength. A comprehensive overview of methodological aspects regarding subject recruitment, organization and conductance of the EVE-study is presented in *paper four* of the present thesis and in another paper addressing methodological aspects of the EVE-study (5).

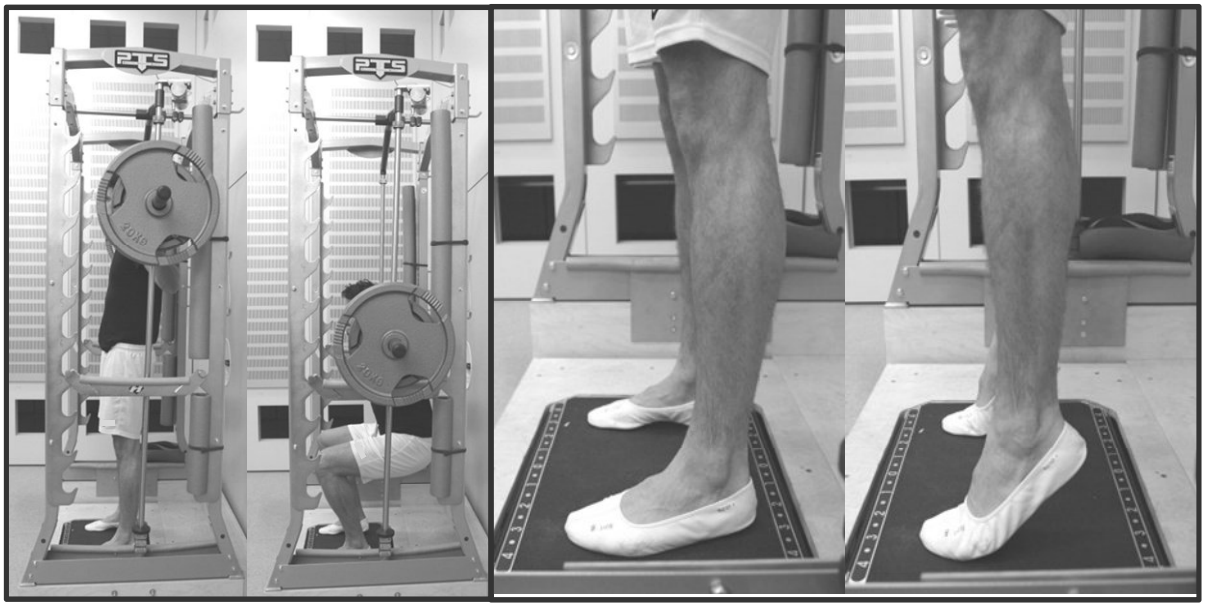

Figure 4. The EVE training set-up. The two pictures on the left hand side depict the correct squat execution while the two pictures on the right hand side illustrate the execution of heel raises.

2.4 Outline of the thesis

It is generally accepted that structure and function of arteries constitute a major factor for overall cardiovascular health and that certain parameters for arterial structure and function can serve to predict cardiovascular risks (93). Given mechanical stress as a major driver for structural and functional adaptations of the vasculature (55), the present thesis sought to elaborate on the specific role of gravity-induced impact loading as a potential source for mechanical signals inducing vascular conditioning. Thus far, studies investigating structural and functional vascular adaptations to either muscle disuse or exercise training have more or less ignored a potential effect of gravitational loading. In these studies, vascular adaptations are always linked to changes of intrinsic hemodynamic forces caused by blood flow and blood pressure (9; 10; 24; 25; 55; 86; 88; 89; 105). Yet, with respect to our 1 g environment on the surface of our planet, there must be a considerable number of gravity induced impacts for a normally active human being (101), inducing mechanical vascular stress, particularly in the vasculature of the lower extremities. In order to investigate the vascular adaptations induced by such a stress, two clinical interventional studies have been conducted where gravity-induced impact loading plays a central role. Within the scope of the HEPHAISTOS study, it was the task to investigate the specific role of habitual gravity-induced impacts upon arterial adaptations. In doing so, a novel device for muscle unloading has been developed and was applied that allows the investigation of vascular disuse adaptations without altering the influence of habitual gravitational loading, which is not achieved in established disuse models like bed rest, limb immobilization, paralysis or space flight. It is known that physical inactivity has a direct conditioning effect on vascular structure and function and it is also known that these mal-adaptations correlate with cardiovascular risk (93). Thus, the study of the effects of gravity-induced impact loading on vascular adaptation during muscle disuse is promising to add to our current knowledge of cardiovascular degradation. Moreover, the relationship between muscle perfusion and exercise tolerance after long-term muscle unloading were elaborated in the present thesis. The question whether functional exercise blood flow and consequently exercise tolerance are impaired after prolonged local muscle disuse has not been considered thus far and was addressed in this thesis. Finally, the impacts of resistance training combined with WBV vibrations upon vascular adaptations were investigated within the framework of the present thesis. It is known that exercise training contributes to cardiovascular health (50), however, it is not known if vibrations, when

superimposed to conventional resistance training, will increase the conditioning effect of muscle work alone. This topic has so far not been addressed and it is of particular interest as WBV as an exercise modality has found its way into various gyms, rehabilitation centers and is even being considered as a potential countermeasure in human space missions (74).

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3 Chapter Three – Scientific papers

3.1 Paper 1: The HEPHAISTOS Study – Protocol and Implementation

Title: The HEPHAISTOS study: Compliance and adherence with a novel orthotic device for calf muscle unloading

Journal: Journal of Musculoskeletal and Neuronal Interactions (accepted for publication)

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Running head: The HEPHAISTOS Study: Protocol and Implementation

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Abstract:

The present manuscript seeks to discuss methodological aspects regarding the application of the novel unloading orthosis 'HEPHAISTOS' that has been specifically developed to study physiological effects of muscular unloading without altering the impact of gravitational loading. The 'HEPHAISTOS' has been applied in an ambulatory clinical interventional study. During gait, the 'HEPHAISTOS' significantly reduces activation and force production of calf muscles while it completely retains body mass-related force on the tibia. Eleven healthy male subjects participated in the study and followed their normal everyday lives while wearing the orthosis. Several measurement sessions have been performed to investigate the time course of structural and functional adaptations during intervention and recovery. Follow-up measurements were performed for one year after the intervention. In consideration of the experiences of a unique ambulant unloading study, organizational and methodological recommendations are discussed in this manuscript. Activity monitoring data obtained with portable accelerometers reveal unchanged gait activities and good subject compliance throughout the intervention. Moreover, electromyography (EMG) and motion data investigating gait properties on reambulation day are illustrated. These data show that during the initial steps following removal of 'HEPHAISTOS', gait was significantly asynchronous indicating an acutely altered motor control in the unloaded lower leg muscles.

Key words: Ambulant unloading intervention, Gravitational loading, Muscular unloading, Orthosis, Hephaistos

Introduction

It is well known that mechanical strain acts as a major driver for tissue adaptations, and the principles of mechanotransduction and mechano-adaptation have been described in various organ systems (4; 8; 11). Of note, the mechanical forces vary within the human body, and musculoskeletal forces are a function of muscle contractions and mass acceleration, often in relation to gravity.

Bone particularly is thought to be sensitive to mechanical strain, and specific yet un-identified force regimens are needed to maintain bone size and strength[8]. Several strings of evidence suggest that larger strains, and also larger strain rates, are more effective to stimulate bone accrual than smaller strains and strain rates (13; 18). Biomechanical analyses suggest that muscle contractions are the origin of the greatest bone forces even in the leg (12), and it has been suggested that skeletal muscle drives skeletal development and maintenance (15). However, there is currently no direct evidence to undermine the notion of bone being a slave of the musculature. Similar arguments may be applied to other tissues that follow the principle of mechanotransduction (9; 19). For example, arteries adapt to blood flow-driven shear forces that act on the endothelial layer (2; 10) and mechanical signals are thought to play a key role in muscle growth[4]. In all of these examples, our knowledge of the specific regimens that drive the adaptive processes *in vivo* is very limited. This is mostly because, origin, magnitude and frequency of mechanical signals are very difficult to assess in the human body, given the interplay of muscle forces and gravity induced forces as major sources for mechanical strain in our one 'G' environment.

Models like bed rest (14), limb immobilization (3) and space flight (21) allow the study of the physiology of gravitational unloading. Such studies demonstrate that gravitational unloading leads to a plethora of de-conditioning processes in various physiological systems (5; 7; 17). Nonetheless, the above models are inconsistent with regards to the mechanisms by which forces with different origins act on the body, as none of these established models differentiates between muscle- and gravity-induced forces. Accordingly, the isolated role of gravity-induced forces as a precursor of physiological adaptations has to date not been addressed in a clinical study.

The aim of the present project was therefore to specifically investigate the effects of muscular unloading and thus the effects of greatly reduced muscular forces, whilst maintaining normal gravitational loading patterns, upon bone, muscles, tendon, blood vessels, cartilage and nervous system in the lower leg. Bone mineral content (BMC) was selected as primary response variable, thus testing the main hypothesis: 'Body weight bearing is insufficient to maintain bone mineral content in the distal human tibia'.

In order to test this hypothesis a novel unloading orthosis (HEPHAISTOS, Fig.1, patent application number: 102011082700.5) has been developed in the German Aerospace Center (DLR) and at the German Sport University. It has been designed to allow normal ambulation and thus body weight application to the skeleton whilst Achilles tendon force is significantly reduced (Ducos et al., manuscript in revision). Eleven healthy male subjects participated in an ambulant study and wore HEPHAISTOS for 56 days (Fig.1). During this time subjects were completely ambulant and followed their normal everyday activities.

The present article presents and discusses data related to design, subject compliance and adherence of and to the HEPHAISTOS study, respectively. It serves the scientific debate of a novel and unique study design, where a new unloading device has been applied. In light of the experiences made during this study, various aspects concerning subject recruitment, measurement protocols, subject compliance and application of the HEPHAISTOS are being discussed. In order to monitor subjects' activity profiles and compliance to the studies requirements, portable accelerometers were used and the results of the activity monitoring are presented in this manuscript. In addition, gait properties of the first steps following removal of the orthosis were carefully obtained and the results provide information on the safety and feasibility of reambulation after unilateral muscular unloading. We hypothesized that normal gait activities would be unchanged during the HEPHAISTOS intervention compared to walking with normal footwear. Furthermore, we expected to observe an asynchronous gait pattern during the initial steps of re-ambulation following 56 days of HEPHAISTOS utilization.

Image 1. The HEPHAISTOS unloading orthosis

Methods

Study characteristics

In the following a methodological overview of the study design is presented. This overview comprises important information on how the study was conducted, including details about sample size calculation, subject recruitment and the actual intervention. Thereafter, methods and technical information of reambulation and activity monitoring measurements are presented. The results of the latter measurements are presented in this manuscript.

Study characteristics: The HEPHAISTOS study (HEP-study) was conducted at the DLR in Cologne, Germany and is registered at *www.clinicaltrials.gov* (Identifier: NCT01576081). It was approved by the Ethics Committee of the Northern Rhine medical association (Ärztekammer Nordrhein, Düsseldorf, Germany). The time frame for the study was scheduled to 14 months, starting on the 19th of April 2011 with baseline data collection (BDC) and completing with the last measurement of the recovery phase on the 5th of July 2012.

Sample size calculation: a sample size calculation has been performed using BMC data from a previous bed rest study (16). The sample size calculation formula has been chosen assuming that the mean sampling distribution of the variable of interest (BMC change) will follow a normal distribution. By estimating the value of the expected mean paired difference and standard deviation of BMC inherent to a period of bed rest of 56 days(18), it was possible to calculate the sample size for this study. For the present ambulant study, we hypothesized that half the loss of bed rest will be sufficient to verify the main hypothesis. Accordingly, the threshold was set to a loss of ≥ 1.7% of tibial BMC. The formula which allows the calculation of the sample size for the comparison of paired means was used:

$$\text{Subjects required} = \frac{(z_\alpha + z_{2\beta})^2 \times \sigma^2}{\delta^2}$$

Where:

z_α is the ordinate of the normal distribution for the first order error probability α

$z_{2\beta}$ is the ordinate of the normal distribution for the second order error probability β

σ^2 is the standard deviation of the paired difference between BDC and R+14 values (the within subject anticipated standard deviation)

δ^2 is the anticipated difference between the BMC values of BDC and R+14.

By accepting a risk of 5% to make an error of the first order (alpha= 0.05) and a risk of 10 % to make an error of the second order (Beta= 0.10; power: 1-beta = 0.9), we obtained from the normal distribution ordinates table:

$$z_{\alpha=0.05} = 1.96$$

$$z_{2\beta=0.10} = 1.282$$

The expected difference between BDC and R+14 BMC values was set to 1.7%. Thus BMC loss was set to 1.7% (δ^2 = 0.017) and the intra subject variability was also set to 1.7% (σ^2 = 0.017) according to data from the literature (17).

The equation becomes then:

$$\text{Subjects required} = \frac{(1.96+1.282)^2 \times 0.017^2}{0.017^2}$$

Subjects required = **10.51**

Subject recruitment: Consequently, 11 male subjects were recruited for the study. The project was promoted by sending email information to previous subjects of the DLR database and to all DLR (Cologne) employees, distributing flyers at public places, hospitals and universities, broadcasting information in the local radio (Radio Köln), advertising the project in an online student job exchange (www.stellenwerk.de) and advertising it on the DLR-webpage. An initial cohort of 93 interested candidates underwent a strict recruitment process including telephone interviews, subject information presentations, medical check-ups, psychological questionnaires (FPI: Freiburger personality inventory), and psychological interviews to eventually identify the 11 suitable candiates. For inclusion into the study, subjects had to be psychologically suitable, medically healthy, aged between 20 and 45 years, in possession of a certificate of good conduct and body mass indexed between 20 and 30. Exclusion criteria were as follows: smoking, professional athletes, diabetes, muscle or joint disease, increased risk of thrombosis (checked via thrombophilia screening), bone fractures 12 months prior to the study, metal implants, any material of osteosynthesis, participation in another clinical intervention study 2 months prior to the study, bleeding disorder, anaesthetic intolerance, vascular disease, epilepsy, claustrophobia, herniated disk, pacemaker, alcohol or drug abuse, anti-inflammatory drug intake, hyperlipidaemia, kidney disease, hyperhomocysteinaemia, vitamin d deficit and chronic back pain. The number of all excluded candidates is listed in Table 1 for each recruitment stage.

Table 1. Subject recruitment

Stage	Total number	Excluded	Invited	Did not attend
Telephone interview	93	15	78	-
Freiburger personality inventory	78	7	24	47
Medical check-up	24	4	20	-
Psychological interview	20	9	11	-

HEPHAISTOS intervention

A coin was tossed to randomly assign the intervention leg. The HEPHAISTOS was manufactured from an orthopaedic technics company (ORTEMA GmbH, Markgrönigen, Germany). The carbon-shaft of HEPHAISTOS was individually tailored for each subject, taking a plaster cast of the lower leg as anatomical template. Two weeks before study start, subjects were familiarized with HEPHAISTOS and final adjustments were made to facilitate a natural gait pattern. The following link leads to the webpage of the DLR with a video clip showing a subject walking with HEPHAISTOS (*http://www.dlr.de/me/en/desktopdefault.aspx/tabid-7389/).* The 11 subjects began the intervention in a staggered order, with three subjects per day over 4 days. Throughout the intervention subjects had to wear HEPHAISTOS during all locomotive activities that required loading of the legs. During this time subjects had to visit the DLR at least once a week for routine examinations, for measurements or for a weekly report. Table 2 depicts the time course of the entire study for one subject including all measurements and events.

Table 2. Study overview. Negative numbers refer to study days before intervention start. Days of baseline data collection = BDC; days of HEPHAISTOS intervention = HEP; recovery phase = R+.

Study day	-52	-46	-34	BDC-14	BDC-8	BDC-7	BDC-3	HEP0	HEP1	HEP2	HEP7	HEP13	HEP14	HEP21	HEP27	HEP28	HEP36	HEP42	HEP49	HEP50	HEP55	HEP56	R+1	R+4	R+5	R+13	R+14	R+15	R+21	R+25	R+27	R+28	R+90	R+92	R+180	R+360
Diet (f=fastend, q=questionnaire, s=standardized, t=tallored)	f	0		s	t	f			t	f	f	q	f		q	f				s	t	f		q		q	f				q	f	q			
Medical check up	x																																			
Psychological interview		x																																		
Plaster cast			x																																	
pQCT				x		x					x		x			x						x			x		x					x		x	x	x
Xtreme CT							x																							x						
HEPHAISTOS adjustment, familiarization				x																																
Galt analysis				x							x		x	x		x	x	x		x		x														
Biopsies				x																x																
Blood samples						x				x			x			x						x			x		x					x		x		
24 hour urine					x				x			x			x						x			x		x				x		x			
Vascular ultrasound						x					x		x			x						x			x		x					x				
Muscle and cartilage MRI																			x									x								
Neuromuscular tests (fatigue and/or MVC)						x																x			x		x		x			x		x	x	x
H-reflex						x																x														
Achilles tendon stiffness								x															x													

Standardized food protocol

As some of the outcome parameters of the study are highly influenced by nutrition, a standardized diet was administered for 4 days on three occasions before taking blood and urine for the measurement of bone markers. Energy intake was calculated for each subject using the subject's body weight. Energy expenditure was calculated by summing the basal metabolic rate (BMR) according to the WHO equation (1) plus 40% of BMR for light physical activity, plus 10% of total energy expenditure (TEE) for energy expenditure associated with thermogenesis from food and beverages. The daily intake of protein (1.2 g/kg BW), fat (< 30% of TEE), carbohydrates (50-55% of TEE), vitamins and minerals matched the German dietary recommended intakes (6). Individual food packages were prepared and packed by a dietician for subjects to take home. Moreover, subjects had to fill out a nutrition questionnaire on six occasions before giving blood and 24H urine samples.

Blood and urine sampling

Blood and urine samples were collected under standardized conditions for bone marker assessment. Fasting morning blood was drawn in supine posture at 7:00 am for each of the sampling days. Once during the baseline collection period (BDC-7), 4 times during the intervention (HEP2, HEP14, HEP28, HEP56) and four times during the recovery period (R+5, R+14, R+28, R+92). Drawn blood samples were immediately centrifuged, aliquoted and stored in a freezer at -20 or -80° respectively their requirements for later analysis. Urine was collected as 24-h urine pools once during baseline collection period (BDC-8), 4 times during the intervention (HEP1, HEP13, HEP27, HEP55) and four times during the recovery period (R+4, R+13, R+27, R+90). Each void was kept dark and cold until final pooling to the 24-h urine pool. Aliquots were stored at -20 or -80°C respectively their requirements for later analysis.

Reambulation measurements

On reambulation day gait properties of the six initial steps without orthosis were investigated using electromyography (TeleMyo 2400 G2 Telemetry System, Noraxon U.S.A. Inc, Scottsdale, Arizona, USA) and a motion capture system (Vicon® Motion Systems Ltd., LA, USA). Both systems were synchronized using a custom-made trigger.

Calculation of step length

Reflective markers were placed on the skin using the standard marker set for the lower body (plug-in-gait skeleton template, Vicon® Motion Systems Ltd., LA, USA). The movement of the marker placed on the metatarsophalangeal joint of the middle toe was then analysed off-line and step length was calculated as the tracked distance between left and right middle toe markers during double limb support of the stance phase.

Electromyography

Surface electromyography (EMG) was recorded using a telemetric device. In order to detect side differences between intervention and contralateral legs, electrodes were placed on both legs applying the *Seniam* recommendations for surface electromyography (www.seniam.org) on following muscles: soleus (Sol), gastrocnemius medialis (GM), tibialis anterior (TA) and vastus lateralis (VL). Electromyographic recordings were obtained using a sampling frequency of 1500Hz. Data were off-line rectified and band-pass filtered (20Hz-500Hz) using

MATLAB (Mathworks, Natick, MA, USA). Thereafter, stance phases were identified using the middle toe metatarsophalangeal joint reflective marker and RMS of the filtered signal was calculated for each stance phase.

Activity monitoring

In order to quantify possible activity changes related to wearing HEPHAISTOS, accelerations (ACCs) of the intervention leg have been continuously recorded during the entire intervention using portable 3-axis digital accelerometers (X1-6A, Gulf Coast Data Concepts, Waveland, USA). Habitual acceleration profiles measured during the week from BDC-14 to BDC-7 were taken as reference. During this period, accelerometers were fixed to the shin using medical bandages (ORTEMA GmbH, Markgrönigen, Germany). During the HEP intervention they were attached to the shaft of the orthosis using Velcro® strips. The X-axis of the accelerometer was aligned with the body longitudinal axis, the Y-axis with the transverse axis and the Z-axis with the sagittal axis. Accelerometers were synchronized with a computer internal clock and set up to automatically start and stop recording all 3-axis accelerations on a built-in SD card every day, from 5.00 am until 12.00 pm, at a 20 Hz sampling rate. A new data file was created for every two hours recording. The data were retrieved on hard disk during the subjects' weekly visit to the DLR using the USB connector the accelerometers were equipped with and the battery of the accelerometer was replaced on that occasion. Acceleration analysis was performed a posteriori using a custom made R program (http://www.r-project.org). Briefly, data were sorted per study week and within each weekly data set, files were pooled according to the time of the day (day^{time}) they were recorded. Thereafter, a moving window over 40 samples with 0-overlap was applied along the pooled data vector obtained for the X-axis, for each day^{time} of each week. For each iteration of the moving window, the standard deviation (SD) of the measured accelerations values was calculated. Each day^{time} acceleration data vector could then be represented by a day^{time} SD vector. Previously, task related mean SDs were identified for activities such as sitting (≈ 0.03 G), standing (≈ 0.01 G) walking (≈ 0.3 G), stair ascending (≈ 0.4 G) and descending (≈ 0.55 G) (unpublished observations). Therefore, the weekly proportion of activity during the HEP study was represented by the counts of SD vector samples equal or superior to 0.1 G in all day^{time} SD vectors in relation to the total amount of SD samples. Moreover, the weekly activity was decomposed into light (ACT^{Light} with 0.1 G < SD < 0.4 G) or heavy (ACT^{Heavy} with SD > 0.4 G).

Statistical analyses

All statistical analyses were performed using using STATISTICA 10.0 for Windows (Statsoft, Tulsa, Oklahoma, USA, 1984-2008). Electromyography and Vicon® parameters were analysed applying a repeated measures ANOVA with *leg* (HEPHAISTOS vs. contralateral) and *step* (step1, step2, step3) as within effect. Accelerometer data were analysed applying the same test, testing effects for *time* for nine study weeks (BDC, W1, W2…W8). Tukey's test has been performed for post hoc analyses. All values are presented as means ± SD and the significance level was set at $P \leq 0.05$.

Results and relevant events during the study

HEPHAISTOS intervention

All eleven subjects completed the 56 intervention days. However, due to reasons which were not related to the study one subject could not attend the HEP56 measurements. All data of this subject which required pre and post comparisons, except the bone parameters, were discarded from further analysis. The EMG reambulation data of one subject had to be discarded from the analysis due to technical failure.

Reambulation

On the last day of the study, after the last HEP56 measurement, subjects made their first steps without HEPHAISTOS. Under controlled conditions subjects were asked to: (1) move the ankle while sitting, (2) stand on two feet, (3) sway, shifting body weight from foot to foot, (4) stand alternately on one foot, (5) sway, from heel stand to tiptoe stand, (6) if possible tiptoe stand on one leg, (7) squat, (8) jump carefully on the spot, (9) walk with assistance, (10) walk alone. Ground reaction forces and centre of gravity motions of movements 1-8 were measured using a force plate (Leonardo Mechanograph®, Novotec, Pforzheim, Germany). During the whole procedure EMG was recorded from soleus, gastrocnemius medialis, vastus lateralis and from tibialis anterior muscles using a telemetric EMG device. In addition gait properties were recorded using the Vicon® motion capturing system. The results of the gait trials (10) are presented below. On reambulation day a professional physiotherapist treated each subject for 60 minutes and checked the mobility of the unloaded ankle, which was for no subject considered as a serious counter indication for reambulation. Five days after reambulation one

subject complained about pressure pain in the area of the intervention forefoot and at the dorsum of this foot. Morton's neuroma was diagnosed which was treated conservatively, and it was judged that this was likely to be facilitated by the subject having splay feet. The ailment vanished 12 weeks after reambulation. All ankle plantarflexor torque tests after HEP56 were cancelled for that subject.

Reambulation electromyography

During the initial six steps without orthosis, RMS of the soleus muscle was by 42.7% (SD = 38.3%) significantly (leg: $P = 0.044$) smaller in the HEPHAISTOS leg if compared to the contralateral leg. There was no difference of soleus muscle EMG between steps for either side (step: $P = 0.59$). The RMS of the tibialis anterior muscle was also significantly (leg: $P = 0.024$) reduced by 39.2% (SD = 43.9%) in the HEPHAISTOS leg, with no differences between steps for either side (step: $P = 0.7$). There were no leg or step specific differences between gastrocnemius medialis muscle (leg: $P = 0.38$; step: $P = 0.87$) and vastus lateralis muscle (leg: $P = 0.25$; step: $P = 0.13$) RMS.

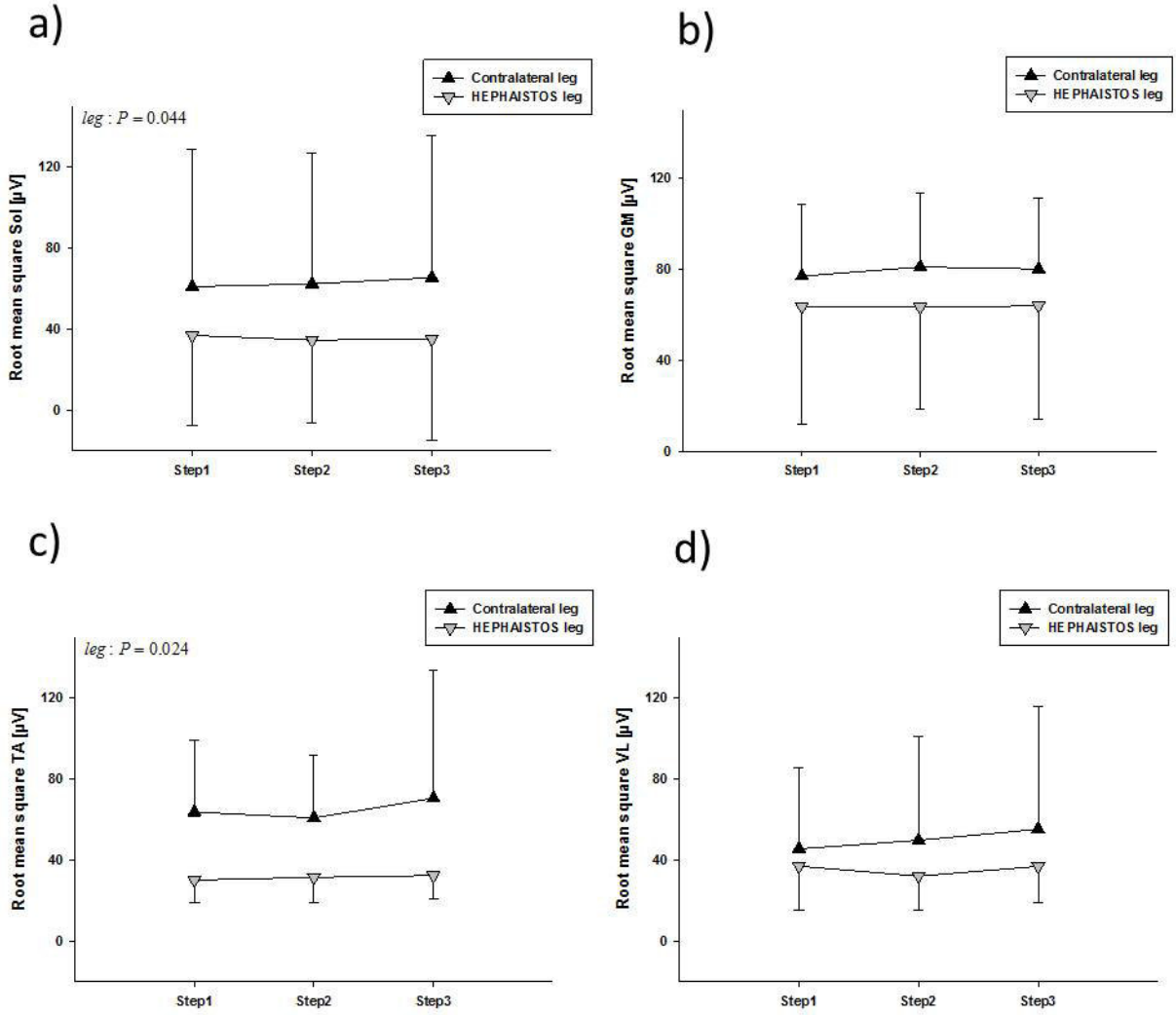

Figure 2. Reambulation electromyography. Electromyographic data of the initial six steps after removal of HEPHAISTOS were obtained for the soleus muscle (Sol), the gastrocnemius medialis muscle (GM), the tibialis anterior muscle (TA) and the vastus lateralis muscle (VL). Root mean square values were calculated for each stance phase and are expressed for the three initial steps on either side. Soleus muscle RMS (P = 0.044) and TA RMS (P = 0.024) were significantly lower on the HEPHAISTOS leg.

Reambulation step length

The step length of the HEPHAISTOS leg was by 16% (SD = 16%) significantly (leg: P = 0.012) shorter compared to the step length of the contralateral leg. There was no length difference between steps (step: P = 0.21).

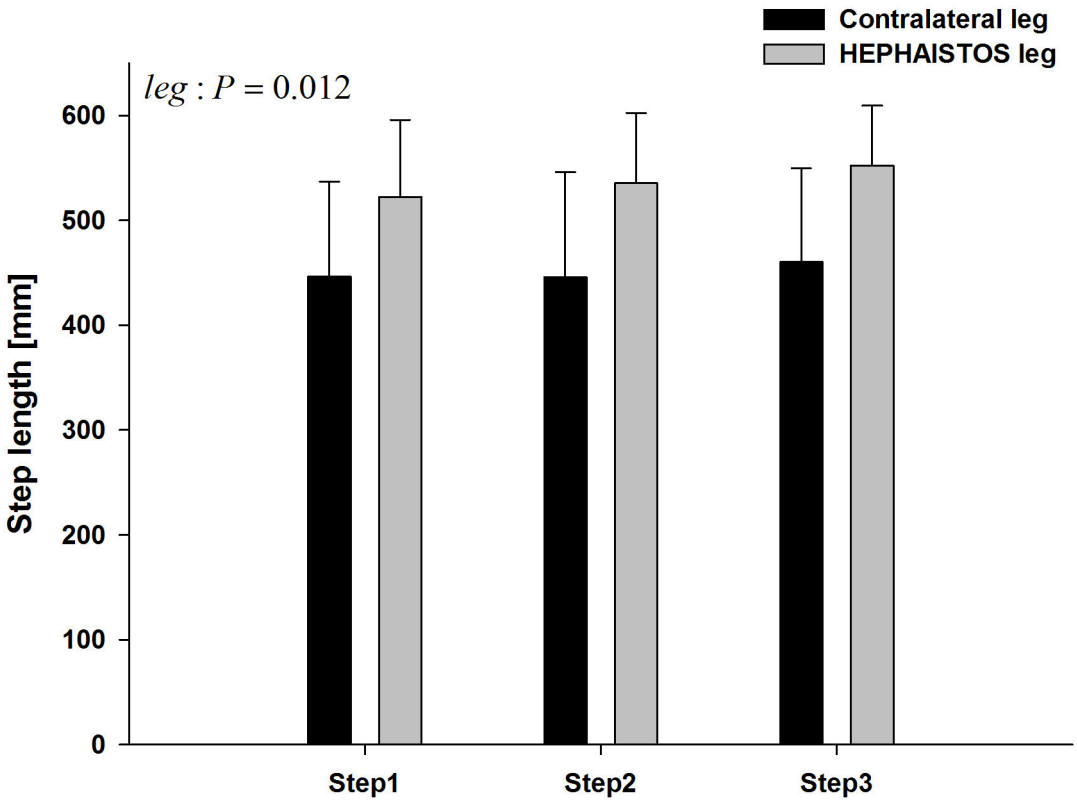

Figure 3. Reambulation step length. Step length was assessed using the Vicon® motion capturing system. One step was determined as the distance between left and right middle toe markers during double limb support of the stance phase. For the initial six steps after removal of HEPHAISTOS, steps were significantly (P = 0.012) shorter on the contralateral side.

Accelerometer activity monitoring

The percentage value of the accelerometer recordings that have been assigned to activity (ACT^{Total}) has decreased significantly over time (P = 0.023). Post hoc testing revealed a significant decrease of ACT^{Total} from 9.7% (SD = 4.5%) at BDC to correspondingly 6.7% (SD = 3%; P = 0.04), 6.7% (SD = 2.5%; P = 0.04) and 6.4% (SD = 2%; P = 0.02) for intervention weeks five, six and seven. The percentage value of recordings that have been assigned to ACT^{Heavy} has also decreased over time ($P < 0.001$). Post hoc testing revealed a significant decrease of ACT^{Heavy} ($P < 0.001$) from 2.7% (SD = 1.5%) at BDC to values below or equal to 1% for all intervention weeks. The percentage value of the recordings that have been assigned to light activities remained unaffected throughout the study (P = 0.5).

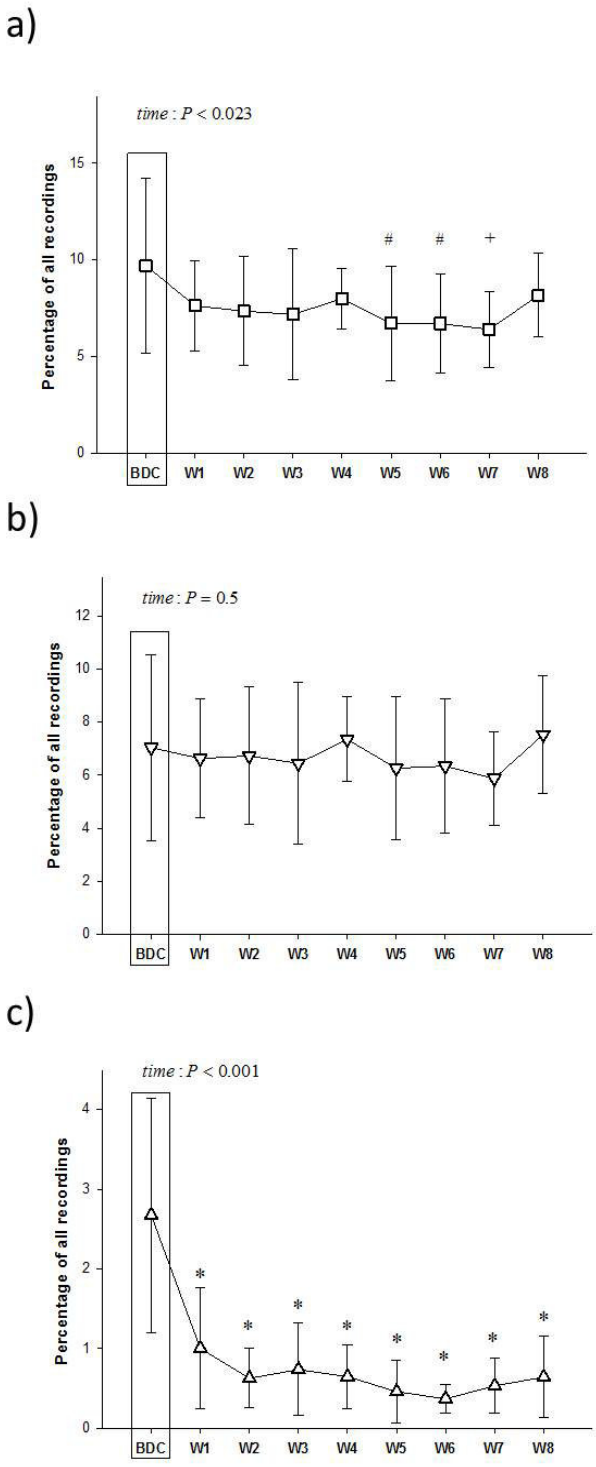

Figure 4. Activity monitoring. Subjects' activities were monitored throughout the entire study (BDC = baseline data collection; W1, W2…W8 = intervention weeks) using portable accelerometers that were fixed to the lower leg. Panel (a) depicts the percentage value of all acceleration recordings that were assigned to general activity (ACTTotal). Panel (b) shows the percentage value of the recordings that were assigned to 'light' activities (ACTLight, e.g. gait) and panel (c) depicts the percentage value of the recordings that were assigned to 'heavy' activities (ACTHeavy, e.g. jumping, running). #P = 0.04, +P = 0.02, *P < 0.001.

CT-measurements

All CT-measurements were approved by the Federal Office for Radiation Protection, Berlin, Germany. Two x-ray devices were being applied in the present study. A high resolution Xtreme CT- scanner (Scanco, SCANCO Medical AG, Brüttisellen, Switzerland) for measurements at BDC-3 and R+25 and a peripheral quantitative tomographic scanner (XTC 3000, Stratec Medizintechnik), for all other bone density measurements. The Xtreme CT scan was conducted in the University clinic in Erlangen, Germany, which 3 subjects could not attend. The overall mean radiation dose for each subject and all measurements was 0,035356 mSv.

MRI-measurements

The cartilage measurements required administration of the contrast agent Gadopentetic acid (Magnevist ®). Two subjects showed symptoms of intolerance and were excluded from this measurement. One subject refused the procedure.

Standardized nutrition, blood and urine sampling

All samples could be collected as planned. The results of those measurements will be published together with the data for the long term bone adaptation (Ducos et al, in preparation).

Discussion

Scientific relevance

The significant reduction of mechanical stimuli as a consequence of gravitational unloading under conditions like bed rest, SCI or space flight is considered as a major source for bone loss (20). For all of these conditions muscular unloading is combined with a change of gravity effects. Exercise interventions as applied for instance in bed rest, that try to compensate for the extensive reduction of muscle work, have only been partially efficient to counteract bone loss (17). However, these interventions do not consider a potential role of gravitational acceleration. The investigation of the effects of the earth attraction force independently from the effects of muscle contraction forces is a logical complement to previous studies and the results of such a novel study approach greatly add to the current knowledge of bone adaptation (Ducos et al., in preparation). Moreover, the application of HEPHAISTOS offers a novel and unique possibility to study the specific effects of gravitational accelerations upon functional and structural adaptations of the other investigated organs (22).

HEPHAISTOS intervention

The intervention with the novel unloading device was very satisfactory. All eleven subjects completed the 56 intervention days without serious complications. Occasionally occurring pressure spots could be successfully counteracted using cushioning and re-adjusting the elastic foot of HEPHAISTOS (see Fig.1). The weak point of the HEPHAISTOS was the anti-slippery sole glued under the carbon prosthetic foot, which showed signs of premature wear during the second week of the intervention. However, selection of a superior material allowed the second sole to outlast the resting intervention time. Our experiences with the novel device made us confident enough to further pursue its application in upcoming clinical trials.

Ambulant study design and subject compliance

The major challenge of an ambulant study design is that subjects cannot be monitored for the biggest part of the intervention. To wear the HEPHAISTOS for 56 days during all daily activities required a great amount of motivation and mental strength from the subjects. Several compliance strategies were developed to ensure that all subjects would use the HEPHAISTOS as stipulated and that all subjects would complete the 56days: (a) Psychological questionnaires (FPIs) and the psychological interviews were applied to

optimize our subject selection. The psychological assessment helped us to find reliable subjects who had a real interest in the topic and perceived the participation in our study as an interesting experience. The above combination of psychological subject characteristics was in our opinion crucial for a successful study. (b) The application of accelerometers to monitor subject activity throughout the entire study provided a certain control tool. The data obtained through accelerometry reveal that subjects wore the HEPHAISTOS orthosis throughout the entire study as the fraction of 'light' activities e.g. gait was comparable between BDC and all other intervention weeks. The finding that heavy activities were significantly reduced during the intervention can be basically attributed to the function of the HEPHAISTOS. Locomotive activities like jumping or running, leading to high acceleration profiles, are restricted with this device. (c) Social events before, during and after the study created a positive team spirit and a familiar atmosphere between subjects and investigators. They also helped to sustain a working relationship between subjects and investigators. (d) One of the project scientists participated as subject in the study. Thus, potential concerns regarding the intervention and measurement procedures have certainly been diminished. As an indication of good subject compliance the distinct artery adaptations shall be mentioned here, which could be detected for all of the eleven participants (22).

Reambulation

The gait trials on reambulation day clearly indicate that motor control of the HEPHAISTOS leg was acutely impaired for the initial steps without orthosis. When wearing HEPHAISTOS, the soleus muscle is primarily impacted (Ducos et al., manuscript in revision) and it therefore appears logical that muscle activation of this muscle is acutely impaired after 56 days HEPHAISTOS intervention. During the initial trials with HEPHAISTOS it seemed that muscle activation of the TA was less affected, however, reambulation data suggest that over the 8 intervention weeks, subjects learned to deactivate the dorsi flexor muscle as TA RMS on the HEPHAISTOS side was significantly decreased during the initial steps. Gastrocnemius medialis and vastus lateralis muscle activity seemed to be less impacted after the intervention, however, this is not unexpected as the HEPHAISTOS orthosis was developed to primarily reduce soleus plantar flexor torque production during gait. The apparent acute imbalance of muscle activation resulted then in an asynchronous gait pattern and it happened that step length of the control leg was significantly reduced. This altered gait pattern can most likely be attributed to a reduced stance time of the HEPHAISTOS leg. Nonetheless, gait properties

normalized quickly and except for the subject suffering from Morton's neuroma, all subjects showed normal gait properties within a few days after reambulation.

Conclusion

In summary, one can conclude that the HEP-project was very successful. The present study adds important knowledge to our current understanding of physiological adaptations induced by muscle unloading. Although the asynchronous gait properties on the first day after removal of the orthosis, the application of HEPHAISTOS had no major side effects and all subjects recovered completely within the time frame of the study. Morton's neuroma, probably as a result of splay foot may be a complication during recovery from wearing the HEPHAISTOS orthosis, and it might also be expected after immobilisation with other models. Although there is no direct proof, activity monitoring data and several other strands of indirect evidence suggests that subjects' compliance with the protocol was very good, thus underlining the feasibility of ambulant immobilisation studies.

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3.2 Paper 2: Gravitational accelerations and arterial adaptation

Title: The specific role of gravitational accelerations for arterial adaptations.

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Running head: Gravitational accelerations and arterial adaptation.

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Abstract

Background: It is mostly agreed that arterial adaptations occur, among others, in response to changes in mechanical stimuli. Models like bed rest, spinal cord injury or limb suspension have been applied to study vascular adaptations to unloading in humans. However, these models cannot distinguish the role of muscle contractions and the role of gravitational accelerations for arterial adaptation.

Methods: The HEPHAISTOS orthosis allows normal ambulation, while it significantly reduces force generation in the lower leg muscles. Eleven subjects wore HEPHAISTOS unilaterally for 56 days and were followed up for another 4 weeks. Arterial diameters, intima media thickness (IMT), flow mediated dilation (FMD) and resting blood flow (BF_{rest}) were measured using high frequency ultrasonography. Arterial adaptations were investigated in the superficial femoral artery (SFA), in the brachial artery (BA) and in the carotid artery (CA).

Results: Mean SFA resting diameter was decreased from 6.57mm (SD = 0.74mm) at baseline to 5.77mm (SD = 0.87mm) at the end of the intervention ($P < 0.001$), while SFA wall-to-lumen ratio, SFA BF_{rest} and SFA FMD remained unaffected throughout the study. The application of HEPHAISTOS had no effect upon structure and function of the systemic control sites, the BA and the CA.

Conclusion: Our findings highlight the importance of muscular contractions for arterial diameter adaptations. Moreover, we propose that FMD and wall-to-lumen ratio are unaffected by ambulating with the HEPHAISTOS orthosis, which is suggestive of habitual acceleration profiles in the lower leg constituting an important stimulus for the maintenance of FMD and wall-to-lumen ratio.

Key words: Gravitational Impacts, Arterial Structure, Arterial Function.

Introduction

It is generally accepted that blood vessels, including larger arteries adapt their structure as well as their functioning in response to alterations in their environment. In this context it is mostly held, that arterial adaptations occur in responses to mechanical stimuli such as shear rate, which is thought to be a primary load (10), acting on the endothelial layer (14; 29). Evidence suggests that endothelial cells (ECs) are able to sense shear rate as friction and dragging forces which are exerted on the cells of the vessel wall by blood motion *(9)*. Alternative mechanical stimuli for arterial adaptation, which are being considered to be sensed by ECs and vascular smooth muscle cells (VSMC) are muscle shortening-related axial "stretch-stresses", which are thought to stretch the adjacent tissues and blood vessels, and pressure-related circumferential wall stresses (10; 19).

Given the habitual activities in our gravitational environment, there must be four potential sources for mechanical stress, acting on the arterial wall: 1) Muscle contractions, provoking mechanical stretch and compression to the vasculature; 2) Phasic, blood flow related pulsatile shear; 3) Blood pressure as the sum of hydrostatic and hydrodynamic pressure; 4) Gravitational accelerations, induced by ground reaction force impacts.

To date, the specific role of gravitational accelerations on arterial adaptation has not been evaluated independently from mechanical stimuli induced by muscle work. Of note, walking and running is associated with vertical accelerations of up to 10 g (12), meaning that habitual everyday activities are likely to provide acceleration-related stresses on the arterial wall, which are not directly depending on muscle contractions.

Chronic disuse such as bed rest, spinal cord injuries, spaceflight and limb immobilization (*ULLS*) are associated with substantial adaptations of arterial structure and function (2; 3; 5; 26). These disuse models reveal that the general reduction of blood flow, as a consequence of muscular unloading, trigger the extensive arterial adaptations observed in immobilized subjects. However, none of these models is valid to independently investigate the effects of gravitational loading for arterial adaptation, since all established disuse models are characterized by both the extensive reduction of muscle work-related stresses and by the absence of gravitational-acceleration-related stresses. These studies also suggest, that constant

blood pressure changes cannot explain the long term adjustment of arterial diameter and arterial function (2; 3).

Given the considerable number of diseased people who are temporarily or permanently immobilized, the study of the effect of such genuine gravitational forces on arterial structure and function could be very relevant for clinical applications in rehabilitation and prevention. Our current interest in this problem had been stirred by a new orthotic device that greatly reduces calf muscle activity and plantar flexion torque (Ducos et al., in preparation), but maintains gravitational loading of the lower leg. Hence, we ventured to explore possible vascular adaptations that would emerge when wearing this new 'HEPHAISTOS' orthosis for 8 weeks. Using ultrasonography, we measured arterial diameters and intima media thickness (IMT) as structural parameters as well as resting blood flow (BF_{rest}) and flow mediated dilation (FMD) as functional parameters. We hypothesized that, compared to the other, well-established disuse models, retention of habitual gravitational impacts in our new model would attenuate arterial diameter decrease, arterial wall thickening and the disuse-specific increase of FMD.

Methods

Study Design, Intervention and Subjects

The unloading orthosis

A novel unloading orthosis (Fig.1, HEPHAISTOS, patent application number 102011082700.5) has been developed in the German Aerospace Center (DLR) in Cologne, Germany (see Fig. 1). The HEPHAISTOS significantly reduces the activation and force production of the major calf muscles during locomotion activities while it completely retains body mass impacts during the stance phase of the gait. It is applied with an elevated plateau shoe (Fig.1b) on the contralateral leg, without the support of crutches and allows normal ambulation. Its biomechanical function and its unloading effects can be briefly explained by the fact that it reduces the plantar lever arm of the foot by approximately 35%. Consequently, it substantially reduces plantar flexor torque and muscle activation. Also, it allows normal ambulation by compensating achilles-tendon function by incorporating an elastic foot, which stores and releases kinetic energy during the stance phase of the gait. The biomechanical

characteristics of the HEPHAISTOS orthosis have been comprehensively assessed by measuring muscle activation using EMG, by measuring reaction force impacts in and outside the orthosis using force plates and pressure insoles, by measuring plantarflexor torque using pressure insoles and by investigating gait characteristics using the a motion capture system. A detailed biomechanical description and analysis of the HEPHAISTOS will be published elsewhere (Ducos et al., in preparation). The following link leads to the webpage of the DLR showing a video with a subject walking with the HEPHAISTOS (*http://www.dlr.de/me/en/desktopdefault.aspx/tabid-7389/*).

Figure 1. The HEPHAISTOS unloading orthosis. A subject wearing HEPHAISTOS and the elevated plateau shoe on the contralateral leg.

The HEP-study

The HEP-study has been registered at *clinicaltrials.gov* (Identifier: NCT01576081). It was designed as an integrative one group ambulatory interventional study where diverse physiological parameters were assessed. The intervention time was scheduled to 8 weeks in

order to enable a valid comparison with previous measurements (3). Eleven male subjects were recruited to wear the HEPHAISTOS unloading orthosis unilaterally. All subjects had been examined by a medical doctor before study inclusion. They also had to pass a psychological assessment including a standardized personality test (FPI: Freiburger personality inventory) and a 45 minute interview with two psychologists specialized in selecting flight personnel and study subjects. Exclusion criteria were: Any known disease or abnormality; any bone, tendon or muscle injury during the last 12 months; smoking; regular strength training; any regular medication. A written informed consent was obtained from all subjects before commencement of the study. The HEP study was approved by the Ethics Committee of the Northern Rhine medical association (Ärztekammer Nordrhein, applilcation number 2010169) in Duesseldorf.

A one € coin (Bundesbank, Cologne, Germany) was tossed for each subject to determine which leg should be unloaded. The 11 subjects were familiarized with their individually adjusted orthosis one week before the intervention started. The familiarization took approximately one hour and was completed as soon as the subjects learned to walk naturally with the orthosis. For the 8 intervention weeks, subjects followed their normal everyday activities while wearing the device in all activities that required loading of the leg. Subjects had to visit the lab on a weekly basis for measurements and reports. After consulting the subjects we estimated a “net wearing time” of 12-16h per day, depending on their habitual activities. The anthropometric data of the subjects at baseline are presented in Table 1.

Table 1. Subject characteristics of the HEP-study.

	(n=11)
Age (yrs)	31.1 (±6.4)
Body mass (kg)	81.2 (±10.0)
Height (m)	1.82 (±0.06)
BMI	24.6 (±2.9)
Systolic blood pressure (mmHg)	119 (±10)
Diastolic blood pressure (mmHg)	73 (±8)
Resting heart rate (beats/min)	64 (±5)

Procedures

Measurement protocol

Arterial diameter of BA and SFA, resting blood flow of BA and SFA, intima media thickness (IMT) of Carotid artery (CA) and SFA, and flow mediated dilation (FMD) of BA and SFA were examined by ultrasonography at baseline, at the 5^{th}, the 28^{th} and the 56^{th} day of the intervention, as well as after 5, 14 and 28 days of recovery (respectively, BDC, HEP5, HEP28, HEP56, R5, R14 and R28).

Measurements

Blood cell velocity and diameter measurements of the BA were performed using the Duplex mode of an echo Doppler device (Mylab25, esaote, Firenze, Italy), with a 12-18 MHz broadband linear transducer (LA 523). Blood cell velocity and diameter measurements of the SFA were performed in the Duplex mode, using a 7.5-12 MHz broadband linear transducer (LA 435). For resting diameter measurements, videos with duration ≥ 1min were recorded for offline analysis. For FMD assessment of SFA and BA, a cuff was placed distal to the probe that was inflated to 300 mmHg for 5 min. 10s prior to cuff deflation video recording was started, and the FMD response was recorded for 5 minutes after cuff deflation. The IMT was determined by the IMT software tool (esaote, QIMT, for MyLab25). The IMT analysis tool processes the radio frequency signal (RF-signal) from the ultrasound device in real time. IMT videos were recorded for ≥ 5 heart cycles, using a 7.5-12 MHz broadband transducer placed parallel to the assessed artery. The region of interest (ROI) for IMT measurements was placed

at the region of the artery with the highest image quality. Resting heart rate and blood pressure were measured before cuff inflation using an electronic sphygmomanometer (medicus pc, boso, Jungingen, Germany).

Subjects rested in a darkened room for at least 20 minutes in supine posture, fastened prior to the measurements for ≥ 8h and also refrained from caffeine, alcohol and exercise for ≥ 8h before the measurement. All measurements were performed by the same examiner. To avoid circadian variation, all measurements were performed at the same time of the day.

The angle of inclination for all Doppler velocity measurements was consistently adjusted to 60°, whereas the vessel area was set parallel to the transducer. The same placement of the probe for all conditions was assured by marking the skin above the artery of interest using anatomical landmarks, such as the upper patella edge for SFA, and the radius epiphysis for the BA. All duplex videos were recorded on an external computer, using the analogue output of the device with a video grabbing system (GrabsterAV 450MX, Terratec, Nettetal, Germany) and an analogue to digital transformation software (MAGIX, Terratec, Nettetal, Germany).

Data processing

Diameters and Flow Mediated Dilation

All videos were analysed off-line. Duplex video analysis was performed using a custom-build edge detection and wall tracking software (Vasculometer 1.2, (4). The signal from the wall tracking software was processed with MATLAB (Mathworks, Natick, MA, USA), using a moving average filter with a span of 500 video frames. The median of all processed values before cuff deflation was taken as resting diameter. The highest value of the filtered signal was identified and used as peak diameter after cuff release. The FMD response was then expressed as the relative increase from resting diameter before cuff inflation to peak diameter after cuff deflation. *Intima Media Thickness:* IMT video analysis was performed using a video sequence of ≥ 5 heart cycles. The IMT videos were analysed offline and the IMT value with the lowest standard deviation (≤ 20µm), which was calculated by the QIMT-software tool from esaote was taken as IMT.

Blood flow

All blood flow measurements are based upon the analysis of Duplex video sequences, using the peak envelope of the Doppler waveform and the arterial diameters. The peak of the envelope of the Doppler waveform and the arterial diameters were automatically detected using the custom-build tracking software (4) and MATLAB software (Mathworks, Natick, MA, USA) to process the tracking signal. The mean velocity (V_{mean}) and the corresponding diameter (D) were then used to calculate blood flow [$BF = (\pi*(D/2)^2)* (V_{mean}/2)*60$], with BF in ml/min, V_{mean} in cm/s and D in cm. Resting blood flow was measured for one minute in supine posture.

Statistical analysis

Statistical analyses were performed using STATISTICA 8.0 for Windows (Statsoft, Tulsa, Oklahoma, USA, 1984-2008). A Repeated measures ANOVA was performed with time (seven levels) as main factor for all FMD, IMT, IMT/lumen, resting diameter, heart rate and blood pressure measurements. Tukey's Test was used for post hoc testing. The results of the blood flow measurements were tested performing paired t-tests. Values are given as means ± SD. The significance level was set at $P \leq 0.05$.

Results

Due to medical reasons which were not related to the current intervention, one subject could not complete the study. The data of this subject are discarded from the analyses.

Diameter

SFA diameter decreased significantly ($P < 0.001$) from BDC to HEP56 by 12.7% (SD = 6.6%). Twenty-eight days after the intervention, SFA diameter reached baseline level again (Fig.2a, $P = 0.92$ for BDC vs. R+28). The intervention did not have an effect upon resting diameter of the BA (Fig.2b, $P = 0.92$).

Intima media thickness and wall-to-lumen ratio

The thickness of intima and media of the SFA changed significantly over time during the HEP-study (Fig.2c, $P = 0.03$). However, post hoc testing did not reveal any significant difference between any particular time points. The ratio between SFA IMT and arterial lumen

remained constant throughout the study (Fig.4, P = 0.19). The IMT of the CA was not affected by the intervention (Fig.2d, P = 0.8).

Flow mediated dilation

No effect of time was observed for the FMD response of the SFA (Fig. 2e, P = 0.32) or for the FMD response of the BA (Fig.2f, P = 0.56).

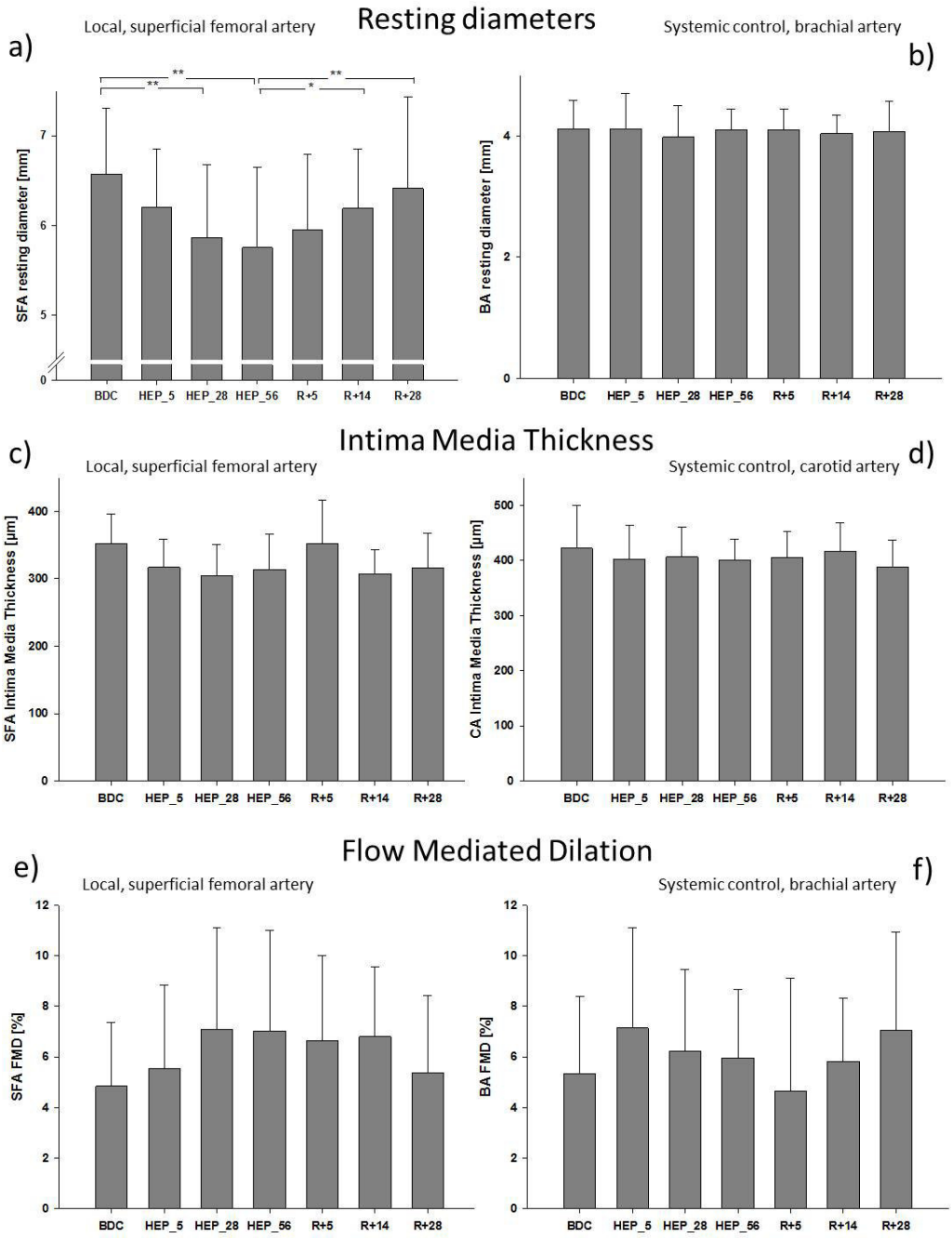

Figure 2. Arterial parameters at rest. Panel a) depicts the time course of SFA resting diameter throughout the study. Superficial femoral artery diameter decreased continuously during the eight intervention weeks. Resting diameter, as measured on the 56th day that the HEPHAISTOS orthosis was worn (HEP56), was 12.7% (SD = 6.6%) lower compared to baseline diameter (P < 0.001). After four weeks of recovery SFA resting diameter went back to BDC level (with P = 0.92 for BDC vs. R+28). Panel b) shows that BA resting diameter remained unaffected. Panel c) depicts the time course of SFA IMT changes. Intima media thickness of the SFA changed significantly over time (P = 0.03). Panel d), panel e) and panel f) reveal respectively, that CA IMT as well as SFA FMD and BA FMD remained unaffected during the study.

Superficial femoral artery resting blood flow

Resting blood flow volume in the SFA remained unaffected after the HEP-intervention (Fig.3c, P = 0.9). The mean resting flow *velocity* in the SFA was significantly increased by 17% (SD = 21.5%) after the study (Fig.3b, P = 0.035), while SFA diameter decreased significantly.

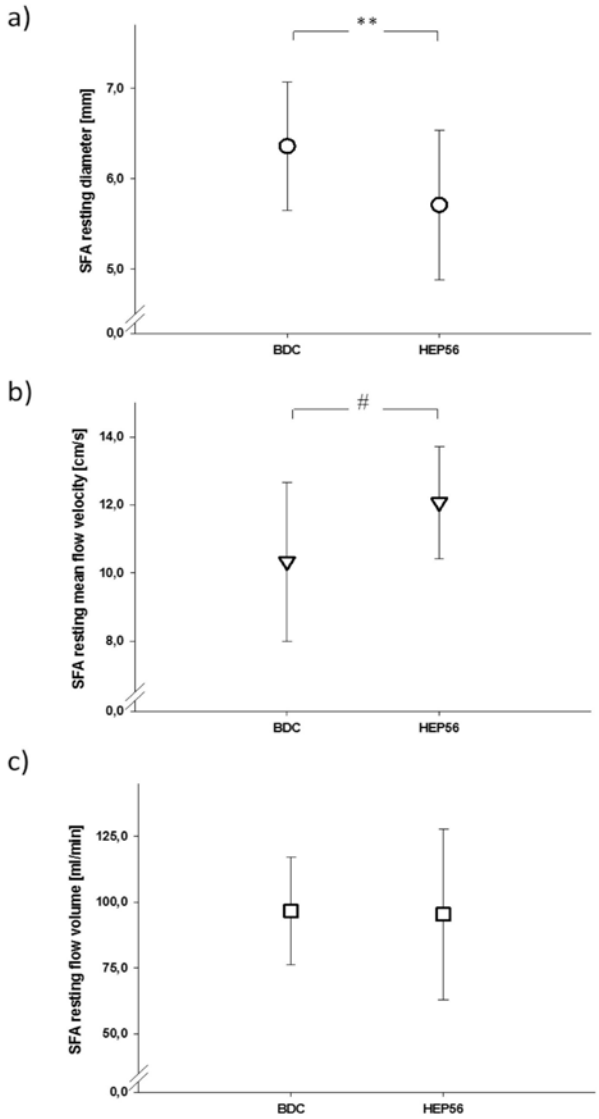

Figure 3. Resting SFA blood flow parameters. SFA resting blood flow volume (c) was calculated using SFA resting diameter (a) and SFA resting mean velocity (b). The unchanged SFA resting flow volume at HEP56 (P = 0.9) results from the elevation of mean flow velocity (+17%, SD = 21.5%; #P = 0.035) while SFA resting diameter decreased significantly (-12.7%, SD = 6.6%; **P < 0.001).

Resting heart rate and blood pressure

Resting heart rate (P = 0.06), as well as systolic (P = 0.27) and diastolic blood pressure (P = 0.2) remained unaffected during the intervention (Fig. 4).

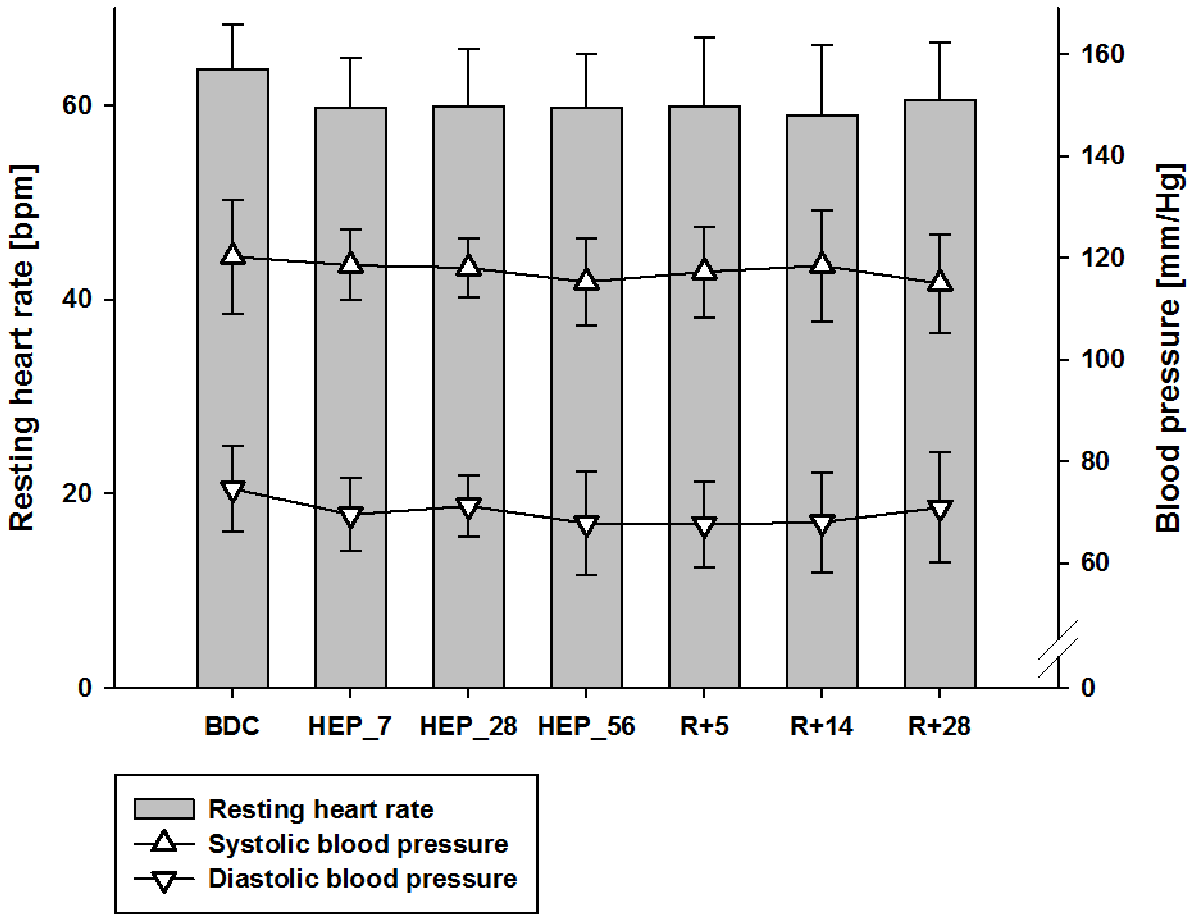

Figure 4. Resting heart rate and blood pressure. Resting heart rate was measured in supine posture before the first cuff inflation. There were no significant changes for all parameters: Heart rate (P = 0.06), systolic (P = 0.27) and diastolic blood pressure (P = 0.2).

Discussion

The main aim of this study was to investigate the independent effects of accelerations during habitual physical activity upon arterial structure and function. For this purpose, we developed a novel unloading orthosis which greatly reduces plantarflexor activation, without reducing ground reaction forces during ambulation, and tested it in a 56day interventional study. The results of the *HEP-study* suggest that gravitational accelerations alone are insufficient to maintain arterial structure. We observed a steady decrease of SFA resting diameter during the unloading phase, which is in line with the literature addressing the effects of the established disuse models (2; 3; 5). However, the present results for IMT, FMD and BF at rest deviate from the above studies, revealing a potential impact of gravitational accelerations on vascular adaptation.

Changes of SFA resting diameter

Our main hypothesis was based upon the view that: a) mechanical stimuli (or the absence of mechanical stimuli) affect the resting diameter in conduit arteries; and that b) habitual gravity-related accelerations would provide an effective stimulus to attenuate arterial resting diameter adaptations when muscle work driven pulsatile shear rate is reduced.

Contrary to these assumptions, the results of this study suggest that the reduction of muscle work and the accompanied reduction of blood flow-related shear lead to a distinct decrease of arterial resting diameter, which is comparable to the diameter reduction in similar time frames under bed rest (Eight weeks: *HEP-study*: -12.7%, SD = 6.6% vs. *bed rest*: 17%, SD = 6.7%, (3) and limb suspension (Four weeks: *HEP-study*: -10.9%, SD = 5.3 vs. *ULLS*: -12%, SD = 4%, (2) conditions, despite the fact that habitual gravitational accelerations remained unchanged in the present study. This finding is in line with the existing literature that supports the idea that blood flow-related endothelial shear acts as the main driver for conduit artery remodelling (1; 13; 29). However, the influence of gravitational accelerations for the resting diameter adjustment of conduit arteries cannot be entirely excluded. Recent findings in bed rest studies suggest that a combination of artificial high frequency accelerations using a vibration plate and resistive exercise can attenuate the immobilisation-induced resting diameter decrease; whereas resistive exercise alone was not sufficient to counteract this

decrease (3; 31). On the other hand, the superposition of vibrations did not have a specific effect in healthy ambulatory subjects, when combined with resistive exercise (33).

A possible way of reconciling the results of the above studies with the results of this study would be to conclude that gravitational accelerations have an impact upon resting diameter adaptations of arteries, if the following two conditions are fulfilled: a) gravitational accelerations have to be applied in combination with muscle contractions (3; 31); and b) the effect of gravitational accelerations must not be saturated through habitual activities (33).

Changes of SFA intima media thickness

The thickness of the intima and media of an artery is thought to provide an index of sub-intimal thickening and is commonly used as a surrogate marker for preclinical atherosclerosis (7). A thicker intima media layer is strongly associated with an increased risk for cardiac and peripheral vascular events, whereas a smaller IMT is associated with cardiovascular health (23).

However, the underlying mechanisms for arterial IMT adaptations are not entirely understood. Thijssen et al. (23) recently reviewed the considered exercise-specific stimuli for IMT adaptation. As for diameter remodelling too, mechanical haemodynamic stimuli such as shear rate and arterial pressure seem to play a crucial role for changes of IMT. An increase of blood flow-related shear rate is thereby associated with a reduction of IMT (24), whereas chronic increases in blood pressure are associated with arterial wall thickening (22). Apart from these, also systemic, non-haemodynamic stimuli, like vascular tone, sympathetic nervous system activity, oxidative stress and inflammatory processes, seem to have an impact upon arterial wall thickness (23).

As mentioned, the application of the HEPHAISTOS unloading orthosis is characterized by a significant local reduction of muscle force generation, hence by a local reduction of blood flow-related shear rate, while hydrostatic arterial pressure and gravitational loading remain unaltered. Consequently, the latter two characteristics deviate from two other investigated disuse models, spinal cord injury (SCI) and bed rest, which reported a systemic increase of IMT (16; 30).

The fact that IMT was reduced in the SFA, but not in the CA largely excludes the possibility of a systemic effect during the intervention. Conversely, the time course of IMT changes during our and other disuse studies suggest that the observed changes of IMT might be more attributable to changes in vascular tone then to actual atherosclerotic structural remodelling (27).

In light of this consideration one could conclude that the present findings deviate from findings observed in bed rest and SCI. This could be for two reasons: a) the time course of SFA IMT changes represents changes of local vascular tone. Accordingly, gravitational accelerations which are absent in bed rest and SCI, do provide a valid stimulus to reduce vascular tone; b) habitual whole body activities maintain sympathetic nerve activity, hence SFA and CA IMT did not (disuse-specifically) systemically increase during the local HEP intervention.

Arterial wall-to-lumen ratio

The finding of an unchanged arterial wall-to-lumen ratio of the SFA in this study is in stark contrast with bed rest, where wall-to-lumen ratio has been found to increase as a consequence of diameter decreases and IMT increases (30). It could well be, that the provision of habitual whole body activity and the provision of habitual gravitational accelerations lead to adjustments of vascular tone which in turn lead to an equilibrium between arterial wall and arterial lumen (see discussion above).

Flow mediated dilation

Typically, muscular disuse is associated with an increase of FMD, which is being used as a measure for endothelial function (2; 3; 5; 30). Both the larger shear stress stimulus occurring in smaller arteries and an increased sensitivity of smooth muscles to NO are being considered as reasons for an increased FMD after physical inactivity (25). Nonetheless, we found in our study that SFA FMD remained unaffected, while SFA diameter showed a distinct inward remodelling during the unloading phase.

Thijssen et al. (28) recently discovered an interesting interaction between arterial structure and arterial function. They found that arterial wall-to-lumen ratio and FMD- response significantly correlated in the investigated arteries of different size across the body. The idea

is, that a thicker media layer, which consists of smooth muscle cells, would provide an increased dilation potential. Accordingly, one possible explanation for the unchanged FMD of the SFA in the present study could be that SFA wall-to-lumen ratio remained constant during the study. Consequently, a possible conclusion would be that the ratio of arterial wall and lumen is more important for FMD adjustment than the magnitude of shear rate.

Superficial femoral artery blood flow

Notwithstanding the distinct inward remodelling during the present study, SFA resting blood flow volume could be maintained after the HEP-intervention. The retention of SFA flow volume is achieved by the elevation of mean flow velocity. The present findings are in agreement with previous studies of deconditioning, where arterial resting blood flow was found to be unchanged after a period of unilateral limb suspension (2), after SCI (6) and after bed rest (3).

In conclusion, the above findings as well as the findings of exercise studies (8; 20; 33) support the contention of Laughlin et al. (14) that the most important muscle work related signal for endothelial cells is constituted by the increased shear stress due to the increase of regional blood flow to provide working muscles with oxygen. Accordingly, the elevated resting shear rate due to diameter decreases and concomitant flow velocity increases, as observed after muscle unloading does not account for the adjustment of resting conduit artery diameter.

Resting heart rate and blood pressure

Changes of resting heart rate strongly correlate with the magnitude of physical activation. As seen in hypokinesia and exercise training, resting heart rate has been reported to progressively increase due to physical inactivity (11; 17) and to decrease in response to increases of physical activity (21). Physical activity is also thought to decrease arterial blood pressure (32), whereas previous hypokinetic studies reveal diverse blood pressure adaptations (15; 18). However, the finding that resting heart rate as well as systolic and diastolic blood pressure did not change for any time point of the present study suggests that subjects maintained their habitual physical activity during the HEPHAISTOS intervention and during recovery.

Conclusions

In conclusion, eight weeks of muscular lower leg unloading with unchanged habitual acceleration profile led to significant site-specific adaptations in SFA diameter. However, we did not observe a disuse specific increase of wall-to-lumen ratio. Furthermore, the FMD response of the investigated arteries seemed to remain unaffected during the intervention. These findings are at variance with findings in bed rest, *ULLS* and *SCI*, where FMD and diameter were always inversely affected (see Fig.5). Based on these data, we propose that FMD is unaffected by ambulating with the HEPHAISTOS orthosis, which is suggestive of habitual acceleration profiles in the lower leg constituting an important stimulus for the maintenance of FMD.

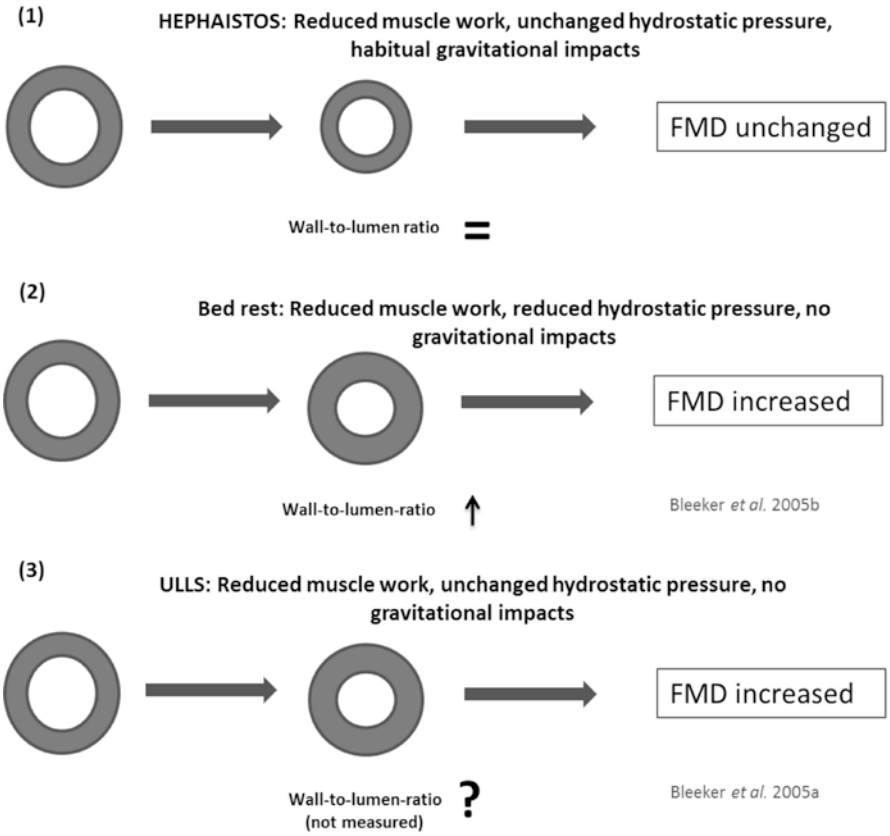

Figure 5. Schematic overview. Simplified illustration of structural and functional SFA adaptations as a consequence of (1) the HEP-intervention, (2) bed rest, and (3) ULLS unloading.

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Conflicts of interest

The authors have no conflicts of interest.

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3.3 Paper 3: Muscle unloading, muscle perfusion and muscle power

Title: The relationship between exercise induced muscle fatigue, arterial blood flow and muscle perfusion after 56 days local muscle unloading

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Running head: Muscle unloading, muscle perfusion and muscle power

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Abstract

In light of the dynamic nature of habitual plantar flexor activity, we utilized an incremental isokinetic exercise test (IIET) to assess the work-related power deficit (WoRPD) as a measure for exercise induced muscle fatigue before and after prolonged calf muscle unloading and in relation to arterial blood flow and muscle perfusion. Eleven male subjects (31 ± 6 years) wore the HEPHAISTOS unloading orthosis unilaterally for 56 days. It allows habitual ambulation while greatly reducing plantar flexor activity and torque production. Endpoint measurements encompassed arterial blood flow, measured in the femoral artery using Doppler ultrasound, oxygenation of the soleus muscle assessed by near infrared spectroscopy, lactate concentrations determined in capillary blood and muscle activity using soleus muscle surface electromyography. Furthermore, soleus muscle biopsies were taken to investigate morphological muscle changes. After the intervention, maximal isokinetic torque was reduced by 23.4% ± 8.2% ($P < 0.001$) and soleus fiber size was reduced by 8.5% ± 13% ($P = 0.016$). However, WoRPD remained unaffected as indicated by an unchanged loss of relative plantar flexor power between pre and post experiments ($P = 0.88$). Blood flow, tissue oxygenation, lactate concentrations, and EMG median frequency kinematics during the exercise test were comparable before and after the intervention, whereas the increase of RMS in response to IIET was less following the intervention ($P = 0.03$). In conclusion, following submaximal isokinetic muscle work, exercise induced muscle fatigue is unaffected after prolonged local muscle unloading. The observation that arterial blood flow was maintained may underlie the unchanged fatigability.

Key words: Muscle unloading, muscle perfusion, muscle power, muscle fatigue

Introduction

Disuse-induced adaptations of skeletal muscle are manifold. Not only is there muscle atrophy and a fibre type shift towards more glycolytic type II fibres with a lower endurance capacity (14; 32), but there are also changes in electromyographic activity (24) as well as distinct structural and functional adaptations of blood vessels supplying the unloaded muscles (31).

As blood vessels are able to rapidly adjust to altered functional demands and considering that peripheral blood flow is dependent on the vasculature, adaptations of structure and function of blood vessels that reduce blood flow must be considered to limit the ability to perform on-going muscle contractions and thus to increase exercise induced muscle fatigability. Muscle fatigue is a general phenomenon that has been previously assessed in different ways (16) and partly explainable as a result of the above adaptations, muscle performance in terms of maximal force output and exercise induced muscle fatigue has indeed been found to be impaired after prolonged disuse (24). The disuse-induced increase of muscle fatigue is, however, not unequivocal, as various studies have found no effect (21; 34) or even a decreased fatigability (27; 28) after muscle unloading. Some parts of the discrepancies between studies may be related to different models of disuse, investigated parameters and exercise protocols. In addition, exercise induced muscle fatigue as studied in previous research (21; 24; 26; 27) was predominantly investigated performing sustained isometric contractions where blood flow is already occluded at comparably low torque levels (13) or performing intermittent isometric contractions (21; 24; 34). These studies did not consider the dynamic nature of the majority of daily locomotive muscle contractions. Other human studies have investigated exercise induced muscle fatigue under dynamic conditions after disuse did not investigate parameters for arterial blood supply and muscle perfusion (3; 15) and final conclusions about the specific impact of blood supply on changes of exercise induced muscle fatigue under dynamic conditions after periods of muscle disuse cannot be made.

Consequently, for the purpose of the present work it should be investigated in how dynamic contractions and moderate work rate would affect muscular power generation after prolonged local muscle unloading. Local exercise induced fatigue was thus assessed calculating the work-related power deficit (WoRPD) during a standardized local exercise test. This test was

specifically developed to reflect habitual calf muscle contractions where a steady blood supply allows for enduring muscle work.

Yet, if reductions in blood supply to locomotive muscles during muscle disuse contribute to dynamic exercise intolerance, this holds great clinical potential to develop effective preventive measures in disease and injury rehabilitation in conditions associated with muscle unloading, aiming at maintaining local circulation (e.g. low intensity exercise or thermotherapy). Therefore, the aim of the present study was to investigate the relationship between blood supply and isokinetic WoRPD after a period of local muscle unloading. Local disuse adaptations in calf muscle blood supply and WoRPD were studied using the HEPHAISTOS unloading orthosis which greatly reduces calf muscle force production during the stance phase without altering the gait pattern (33); Ducos et al., manuscript in revision). Previous whole body (5; 11; 20) and local disuse studies (4; 28; 29) have found that the vasculature adapts distinctly, structurally as well as functionally to unloading. However, these studies did not elaborate on the consequences of the disuse-induced vascular adaptations with regard to exercise induced muscle fatigue in terms of a WoRPD.

It was in light of the above considerations the aim of the present study to comprehensively investigate changes of local blood supply and its potential impact on exercise induced muscle fatigue after prolonged muscle unloading. In order to investigate the functional muscle capacity during an incremental isokinetic exercise test (IIET) that was performed before and after the unloading intervention, isokinetic plantar flexor torque was continuously recorded and muscle power was calculated. Further, neuronal changes after muscle unloading should be detected measuring electromyographic soleus muscle activity during the exercise test, while femoral artery blood flow (ultrasonography), blood lactate concentrations and soleus muscle tissue oxygenation (near infrared spectroscopy) were measured to assess changes of blood supply and metabolic properties of the unloaded muscle. In addition, before and after the HEPHAISTOS intervention, muscle biopsies were taken from the soleus muscle and histochemically analysed to assess fibre type distribution and muscle capillarization.

Thus, the primary hypothesis of the present study was that after 8 weeks of local muscle unloading the local blood flow at a given relative submaximal workload is reduced. We further expected a priori that if blood flow would be reduced, the reduction of blood supply would lead to an increase of WoRPD under isokinetic conditions.

Methods

Participants

Before study inclusion, subjects underwent comprehensive medical and psychological examinations. Prior to commencement of the study, a written informed consent was obtained from all subjects. The HEPHAISTOS study was approved by the Ethics Committee of the Northern Rhine medical association (Ärztekammer Nordrhein, Duesseldorf, Germany).

Procedures

Unloading orthosis

In order to inactivate the calf muscles during locomotion, subjects wore the HEPHAISTOS orthosis in all daily activities that required loading of the legs (Fig.1, patent application number 102011082700.5). The orthosis allows normal ambulation while activation and force production of the major calf muscles are significantly reduced, whereas the impact of ground reaction forces is completely retained. The biomechanical principles and acute effects of wearing the HEPHAITOS are published elsewhere (Ducos et al., manuscript in revision). In short, HEPHAISTOS reduces the plantar lever arm of the foot by approximately 35%, while ground reaction forces are retained. This leads to a substantial reduction of plantar flexor activation and plantar flexor torque production, in particular of the soleus muscle. A natural gait pattern can be maintained through the function of the elastic foot underneath the sole, which stores and releases energy during gait much like the Achilles tendon. The link below leads to the DLR Space Physiology webpage where a video of a subjectwallking with HEPHAISTOS is presented (*http://www.dlr.de/me/en/desktopdefault.aspx/tabid-7389/).*

Figure 1. HEPHAISTOS. A subject wearing the HEPHAISTOS unloading orthosis and the elevated contralateral plateau shoe.

HEPHAISTOS intervention

A detailed description of the *s*tudy design of the HEPHAISTOS intervention will be published elsewhere (Weber et al., manuscript in revision). The study has been registered at *www.clinicaltrials.gov* (NCT01576081). Briefly, the HEPHAISTOS study (HEP-study) was conducted as an integrative single-group ambulatory interventional study. Eleven healthy male subjects (31 ± 6 years) wore the HEPHAISTOS unloading orthosis unilaterally for 56 days, while on the other leg a shoe with an elevated sole of the same height was worn. During the study, participants visited the laboratory for measurements and reports on a weekly basis.

Isokinetic incremental exercise test

An exercise test was performed at baseline data collection (BDC) and on the last day of the intervention (HEP56) that was thought to be challenging but not impossible to complete after 56 days HEPHAISTOS unloading. An incremental exercise design was chosen to ensure valid ultrasound measurements during the moderate stages in order to test the primary hypothesis and to enforce a work-related power deficit following the higher increments in order to test the secondary hypothesis. To allow investigations of WoRPD characteristics independently of changes related to maximal strength losses, submaximal target torque stages were normalized to the current maximal voluntary contraction (MVC) strength. While lying in supine position with the foot attached to a dynamometer (Biodex system 3, Biodex Medical Systems, NY, US), subjects performed four incremental exercise stages that were, based on pilot study results, set to 30%, 40%, 45% and 50% of the current isokinetic maximum voluntary contraction strength (MVC_R), which in turn was assessed prior to the exercise test. Each stage consisted of 40 submaximal contractions, followed by two maximal isokinetic plantar flexor contractions. Foot dorsiflexion was performed passively with external support. Between successive stages, subjects rested for an interval of five seconds. Angular velocity was set to 20 $deg \cdot s^{-1}$ and the total movement angle ranged from -5 deg dorsiflexion to 15 deg plantar flexion, where 0 deg refers to the neutral position. To assess the reference MVC (MVC_R), subjects performed two sets of five maximal contractions per set, with one minute pause between sets. The incremental submaximal stages were then set as a fraction of the MVC_R. During the IIET subjects performed two MVCs before the first stage and two MVCs at the end of each stage. The highest power of the two MVCs at the end of a stage was used to assess WoRPD, given as a percentage power difference from MVC_R. For all MVC assessments, subjects were asked to produce as much plantar flexor torque as possible during verbal encouragement. Real time visual feedback of the produced torque was provided to ascertain correct contraction strength for each submaximal stage. A schematic overview of the exercise protocol, including all measurements, is depicted in Figure 2.

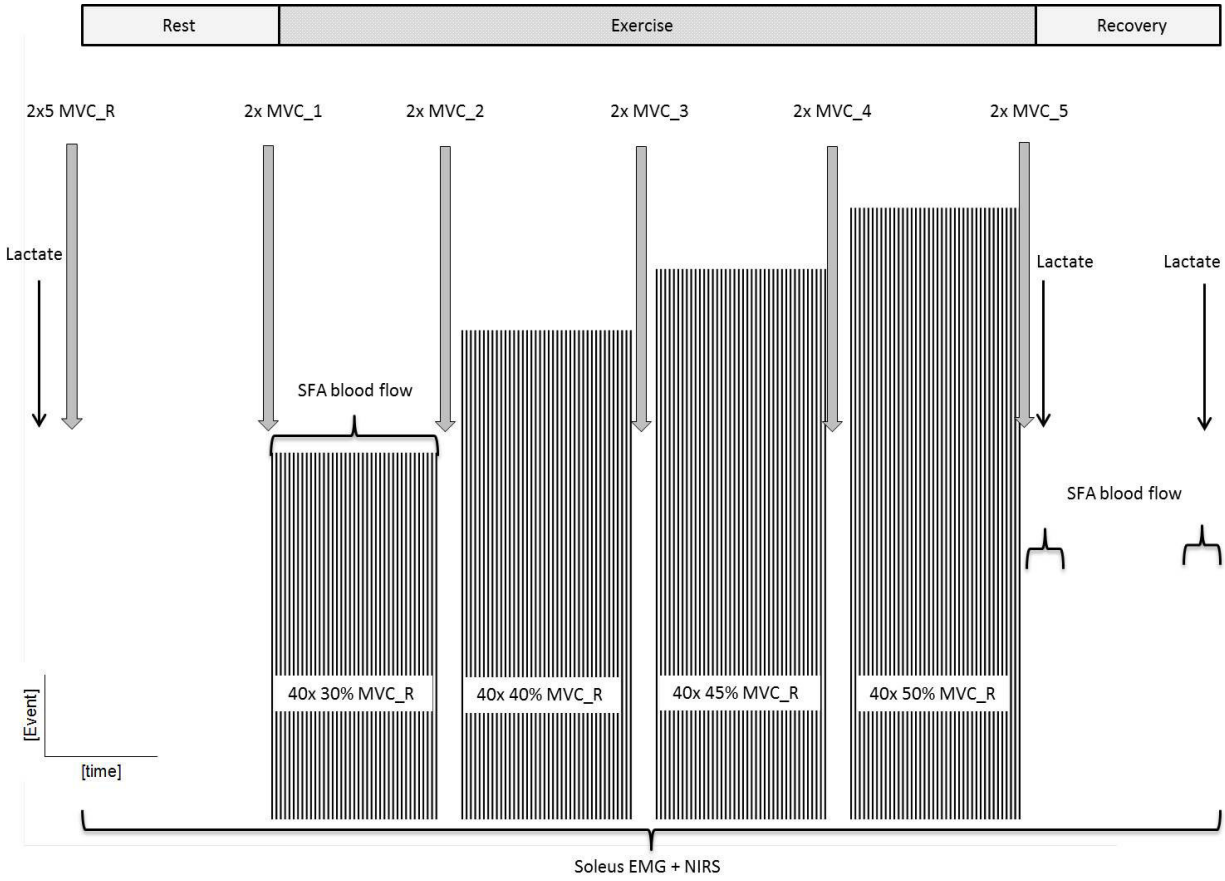

Figure 2. Exercise protocol. Schematic overview of the isokinetic incremental exercise test (IIET) including all measurements that were performed. MVC: Maximal voluntary contraction; SFA: Superficial femoral artery; EMG: Electromyography; NIRS: Near infrared spectroscopy.

Functional measurements

Isokinetic measurements

Plantar flexor torque (τ) was recorded during the entire exercise protocol using the internal software of the Biodex3 dynamometer and a sampling frequency of 100 Hz. Peak torques were then determined off-line for each MVC. Angular velocity (ω) was set to 20 $deg \cdot s^{-1}$ (0.3491 $rad \cdot s^{-1}$) for all torque measurements and mechanical power (P) was then calculated as: $P = \tau \cdot \omega$, with τ in Nm and ω in $rad \cdot s^{-1}$ and P in $Nm \cdot s^{-1}$.

Arterial blood flow

Blood flow (BF) was measured in the superficial femoral artery (SFA) using a Doppler ultrasound device (Mylab 25, esaote, Firenze, Italy) with a 7.5 to 12 MHz broadband linear transducer. Resting blood flow (BF_{rest}) was measured in the morning under standardized conditions: subjects were asked to fast, refrain from alcohol, caffeine and exercise for ≥ 8 hours prior to the measurement. Throughout the IIET, blood flow was measured during the 30% exercise stage ($BF_{exercise}$) directly after the last stage of the protocol (BF_{rec1}) and after two minutes of recovery (BF_{rec2}). The Duplex mode was used to simultaneously measure arterial diameter and blood flow velocity. The angle of inclination for Doppler measurements was set to 60 deg where the probe was placed parallel to the longitudinal section of the artery. Ultrasound videos were recorded on an external computer using the analogue output of the device and a video grabbing system (GrabsterAV 450MX, Terratec, Nettetal, Germany) together with an analogue to digital transformation software (MAGIX, Terratec, Nettetal, Germany). Off-line analysis of the recorded videos was performed applying custom-built software (8). Arterial blood flow was calculated using the envelope of the Doppler signal and the corresponding SFA diameters. Mean flow velocity (V_{mean}) and the corresponding artery diameter (D) were then used to calculate blood flow for each condition as:

$BF = \pi(D \cdot 0.5)^2 \cdot (V_{mean} \cdot 0.5) \cdot 60$, with BF in $ml \cdot min^{-1}$, V_{mean} in $cm \cdot s^{-1}$ and D in cm. Exercise induced dilation was calculated as the relative diameter increase from rest.

Blood supply/mechanical power ratio

The blood flow values ($ml \cdot min^{-1}$) for the 30% MVC stage ($BF_{exercise}$) and the corresponding submaximal plantar flexor power ($Nm \cdot s^{-1}$) were taken to calculate the ratio of blood supply and mechanical power (BF:P), with BF:P in $ml \cdot Nm^{-1}$.

Muscle tissue oxygenation

Near infrared spectroscopy was used during the entire experiment using a custom-made device (RheinAhrCampus Remagen of the Koblenz University of Applied Sciences). This device consists of a slow scan camera (model 7358-0003, Princeton Instruments, Roper Scientific, Trenton, US), a detector chip with 1340x400 pixels and 16 bit resolution, a controller unit (model Spec-10, Princeton Instruments, Roper Scientific, Trenton, US) and a spectrometer (model SP-150, Acton optics and coatings, Princeton Instruments, Acton, US). Details about the mode of operation of this device have been published elsewhere (18). Tissue oxygenation index (TOI) was measured at the distal medial side of the soleus muscle using a sampling rate of 1 Hz. The median soleus muscle TOI was determined from data acquired one minute before the IIET (TOI_{rest}) and for two minutes after the IIET ($TOI_{recovery}$). The minimal TOI was determined using the full period of the incremental test ($TOI_{exercise}$).

Electromyography

Soleus muscle surface EMG was obtained using a telemetric device (Trigno Wireless, Delsys Inc., Boston, US) applying the Seniam recommendations for surface electromyography (www.seniam.org). Electromyographic recordings were obtained throughout the entire IIET protocol using a sampling frequency of 4000 Hz. The signal was off-line rectified and high pass-filtered (>50Hz) with MATLAB (Mathworks, Natick, MA, USA). Submaximal contractions were detected by applying a threshold equivalent to 30 times the standard deviation of the EMG signal at rest. After visual inspection of the signal, incorrectly detected contractions were not considered. Subsequently, root mean square (RMS) and median frequency (MF) were calculated for each submaximal contraction. Values for RMS and MF of missing contractions were interpolated using the Piecewise Cubic Hermite Interpolating Polynomial (pChip function, MATLAB library). Means of RMS and MF were then calculated for all IIET stages (Fig. 7).

Lactate measurements

Blood lactate concentration was assessed in capillary blood taken from the ear lobe before, directly after and 2 min after the IIET protocol (LA_{rest}, LA_{rec1}, LA_{rec2}, respectively). The lactate concentration was analysed using a portable lactate analyser (Lactate Pro, Arkray, Kyoto City, Japan).

Histochemical analysis

Biopsy sampling

Biopsy samples from soleus muscle were collected after overnight fasting, both at baseline and on the 50th day of the immobilization phase in order to assure uncompromised functional data acquisition at HEP56. Biopsies were taken from the lateral side of the muscle, approximately 1 cm below the belly of the lateral gastrocnemius muscle. After skin disinfection and local anaesthesia (2-3 ml of 2% Lidocaine), skin and muscle fasciae were incised for 10 mm and muscle samples were taken with a Weil–Blakely rongeur (Gebrüder Zepf Medizintechnik, Tuttlingen, Germany). Samples were, under rapid shaking, immediately frozen in liquid nitrogen and subsequently stored at -80°C for further analyses.

Lectin staining of capillaries

Ten-µm thick cross-sections of soleus muscle biopsies were cut in a cryostat. Capillaries were stained with lectin (*Ulex Europaeus*): sections were fixed in ice-cold aceton for 15 min and washed in HEPES buffer. Natural occurring peroxidase activity was blocked and after washing in HEPES sections were incubated in lectin solution (50µg/ml in HEPES). The location of the capillaries was revealed with 40 min ABC-staining solution (ABC, Vectastain, Vector Laboratories, Burlingame, US) followed after wash steps, by incubation with DAB (DAB substrate kit, Vector Laboratories, Burlingame, US) and embedded in glycerine gelatine.

Myosin ATPase staining

Serial sections were stained for myosin ATPase according to Brooke & Kaiser (9). Briefly, sections were pre-incubated in sodium acetate solution (pH: 4.35), washed, incubated in alkaline buffer (pH: 9.4), washed, incubated in cobalt chloride solution (2%), washed, incubated in ammonium sulfide solution (1%), washed and mounted in glycerine gelatine. Type I fibres appear dark and type II fibres light (Fig. 3).

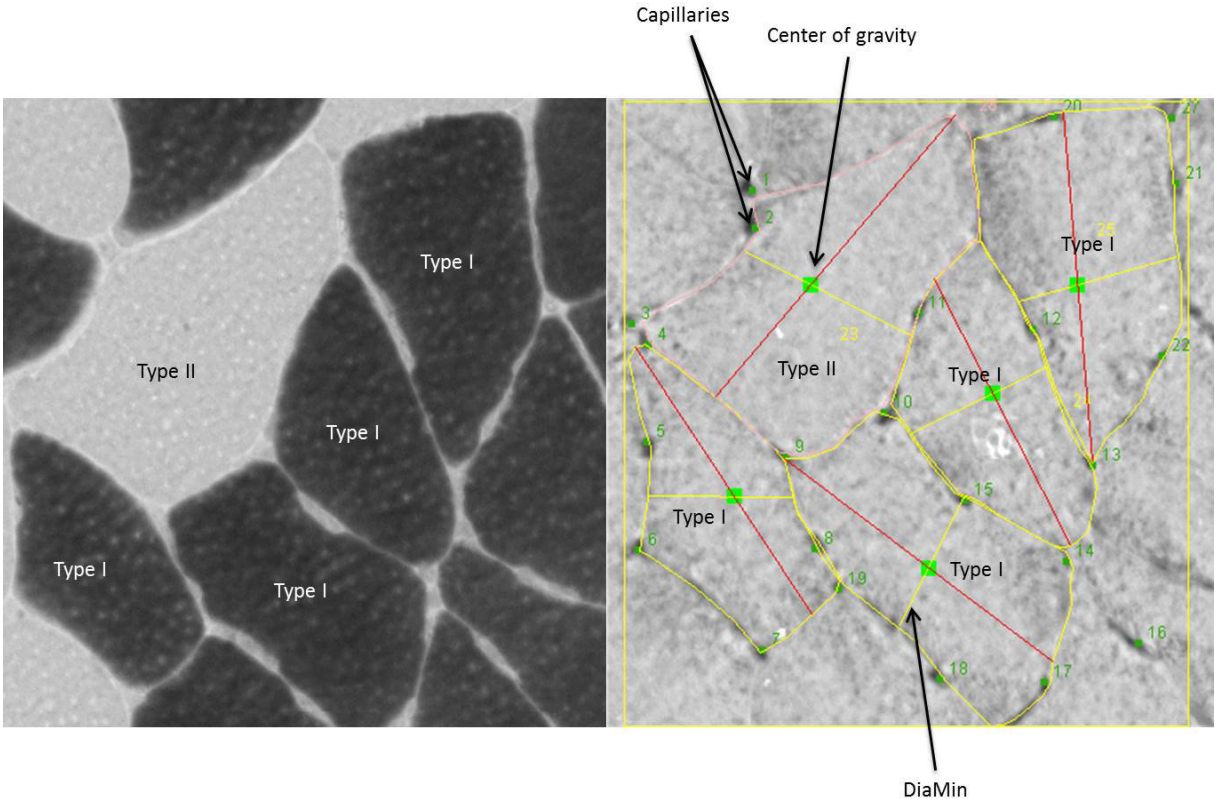

Figure 3. Soleus muscle sections. Left: Myosin ATPase staining. Right: Lectin staining for capillaries. Fibre types were transferred from Myosin ATPase sections. Fibre polygons and capillaries were then analysed using custom-built software. Numbers are assigned for each object by the software. Note that for each muscle fibre DiaMin crosses the polygon centre of gravity.

Analysis of stained sections

Whole sections were photographed with a 20-fold magnification using a light microscope (Axio Scope.A1, Carl Zeiss Microscopy GmbH, Göttingen, Germany) and a USB-Monochrome camera with a 1280x960 pixel chip (ICX205AL, Sony Corporation, Tokyo, Japan). Lectin-stained images were then analysed using the custom-made 'Histometer' software (Fig. 3), which is implemented as plugin into the ImageJ image processing software (ImageJ 1.46r, National Institute of Health, US). Regions of interest (ROIs) were determined in the area of the muscle section with predominantly polygonal or circular shaped muscle fibres. Fibres that were sectioned longitudinally were avoided in the analysis. Based on pixel analyses within a given ROI smallest fibre diameters (DiaMin), fibre cross-sectional areas (FCSA), capillaries around fibres (CaF), capillary density (CD) and capillary-to-fibre ratio (C:F) were determined. DiaMin was calculated as the smallest diameter (in µm) of each fibre-polygon that crosses the polygon centre of gravity (Fig.3), FCSA was calculated as the sum of all pixels within one polygon (in μm^2), CaF was calculated as the number of capillaries that

were in direct contact with the fibre polygon (distance from capillary to fibre < 9.3 µm), CD as the overall number of capillaries divided by the area of the entire ROI and C:F was calculated as the overall number of capillaries divided by the overall number of fibres. Finally, fibre type distribution was assessed as the relative distribution of type I and type II fibres, and fibre area distribution as the relative area occupied by either fibre type. The average number of analysed fibres per ROI and section was 135 (SD = 48). All image analyses were performed by the same investigator.

Statistical analysis

Statistical analyses were performed using STATISTICA 8.0 for Windows (Statsoft, Tulsa, Oklahoma, USA, 1984-2008). A repeated measures ANOVA was performed with four time levels (rest, exercise, rec1, rec2) and two groups (BDC, HEP56) to detect changes in arterial blood flow and exercising blood flow velocity. Exercise induced dilation was tested in the same way with three different time levels (exercise, rec1, rec2). Soleus muscle oxygenation (TOI_{rest}, $TOI_{exercise}$, $TOI_{recovery}$) and lactate concentrations (LA_{rest}, LA_{rec1}, LA_{rec2}) were analysed with three different time levels and two groups (BDC, HEP56). In order to assess changes of MVCs within the IIET protocol, a repeated-measures ANOVA was performed with six time levels (MVCR - MVC5) and two groups (BDC and HEP56). Elektromyography data were analysed with four time levels ($EMG_{30\%MVC}$, $EMG_{40\%MVC}$, $EMG_{45\%MVC}$, $EMG_{50\%MVC}$) and two groups (BDC, HEP56). Tukey's test was used for post-hoc testing. Pre-post differences BF:P ratios as well as soleus muscle biopsy data were analysed with paired t-tests. Values are expressed as means ± SD. The significance level was set at $P \leq 0.05$.

Results

Due to reasons unrelated to the HEPHAISTOS intervention, one subject could not attend the HEP56 IIET. Nonetheless, soleus muscle biopsies of this subject were taken as scheduled and the data were taken into account for soleus muscle morphology analysis. Electromyography data of two subjects had to be discarded from analysis due to technical failure. Superficial femoral artery blood flow could only be measured at rest, during the moderate 30% MVC stage of the IIET and after the IIET and not, as it was initially planned and tested before on experienced investigators, during the entire IIET. Whole body motionartefacts generally precluded sufficient Duplex ultrasound measurements with the relatively inexperienced subjects during higher torque levels.

Calf muscle performance

Absolute reference plantar flexor MVC torque (MVC_R) was significantly ($P < 0.001$) reduced by 23.4% (SD = 8.2%) at HEP56 compared to the BDC value (Fig. 4a). During the IIET, MVC power declined significantly (time: $P < 0.001$) from 49.9 Nm · s^{-1} (SD = 6.8 Nm · s^{-1}) to 36.8 Nm · s^{-1} (SD = 6.7 Nm · s^{-1}) at BDC and from 38.3 Nm · s^{-1} (SD = 6.9 Nm · s^{-1}) to 27.2 Nm · s^{-1} (SD = 4.8 Nm · s^{-1}) at HEP56 (Fig.4b). The IIET-related power reductions on both days were comparable when expressed as per cent decline (group: $P = 0.88$; Fig. 4c).

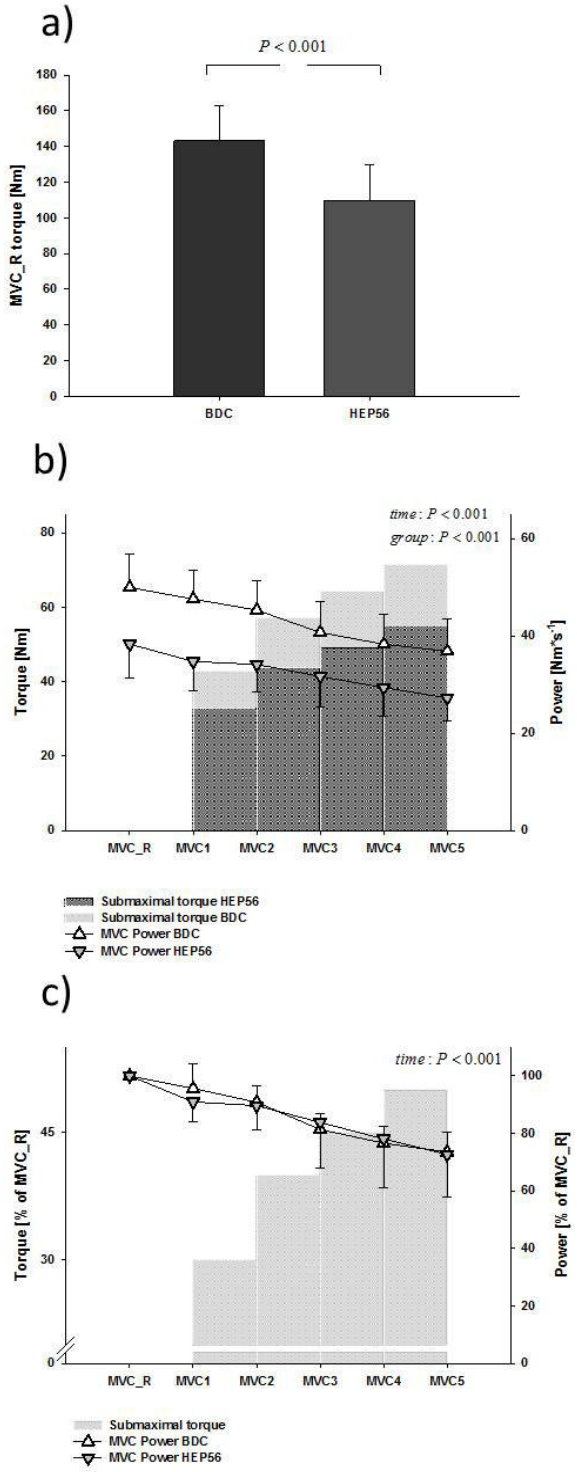

Figure 4. Isokinetic plantar flexor performance. Panel (a) depicts the significant (P < 0.001) decrease of 23.4 % (SD = 8.2%) of absolute peak isokinetic plantar flexor torque (MVC_R) after the intervention. The work related power deficit is depicted in absolute values (b) and as percentage decrease from MVC_R (c). Vertical bars depict submaximal work stages with the corresponding target torques; with MVC_R as reference MVC and MVC1 to MVC5 as MVCs within the IIET. There was no difference of the WoRPD between BDC and HEP56 (P = 0.88).

Arterial blood flow parameters

Blood flow increased significantly in response to the IIET (time: $P < 0.001$) from 96 ml·min^{-1} (SD = 27 ml·min^{-1}) at rest (BF_{rest}) to 250 ml·min^{-1} (SD = ml·min^{-1}) for $BF_{exercise}$ and to 364 ml·min^{-1} (SD = ml·min^{-1}) until BF_{rec2}, *i.e.* two minutes after termination of the IIET. Absolute SFA blood flow did not change after the intervention for any of the four tested time levels (Fig. 5a), as indicated by the absence of a significant group effect ($P = 0.95$). Mean SFA blood flow velocity increased significantly in response to the IIET (Fig.5b; time: $P < 0.001$) with no significant differences between BDC and HEP56 (group: $P = 0.16$). Resting and exercising SFA diameters were significantly smaller at HEP56 (Fig.5b; group: $P = 0.03$) compared with BDC. In response to the IIET, SFA diameter dilated significantly (time: $P = 0.002$) by 5.8% (SD = 7.5%) from rest to two minutes recovery (rec2) for the pooled data of BDC and HEP56. There is trend (group: $P = 0.07$) that HEP56 exercise dilation was more pronounced than BDC exercise dilation.

Blood-supply/mechanical-power ratio

The ratio between SFA blood flow during the 30% MVC stage and the corresponding plantar flexor power (BF:P) increased significantly ($P = 0.0046$) from 0.27 ml·Nm^{-1} (SD = 0.06 ml·Nm^{-1}) at BDC to 0.39 ml·Nm^{-1} (SD = 0.11 ml·Nm^{-1}) at HEP56 (Fig. 5d).

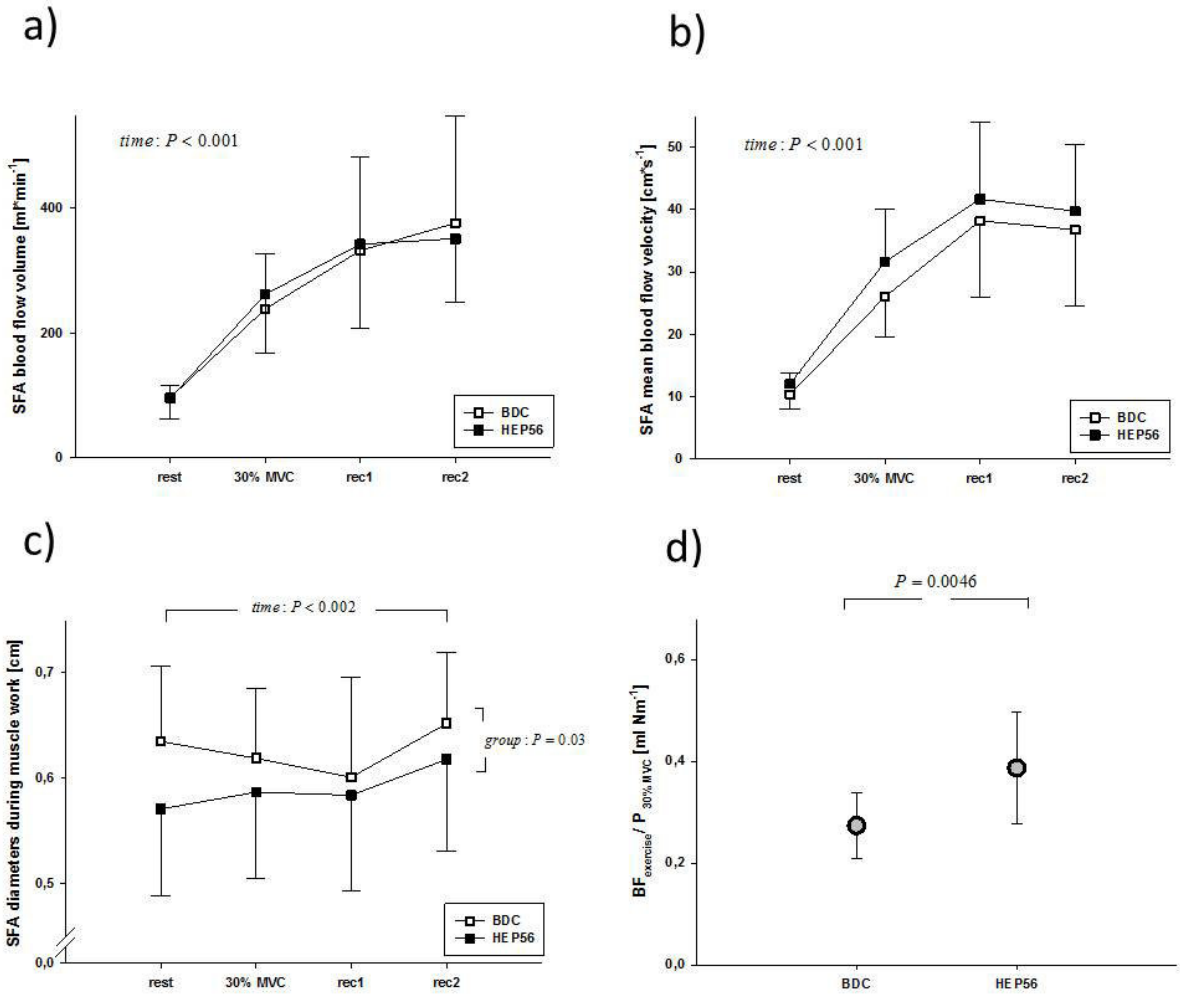

Figure 5. Arterial blood flow. (a) Absolute blood flow was not different between BDC and HEP56 for all conditions (P = 0.95). Within the IIET, blood flow, mean flow velocity (b) and SFA diameters (c) increased significantly over time and absolute arterial diameters were significantly smaller at HEP56 (P = 0.03). Panel d) shows that the ratio of mean blood flow at 30% MVC (〖BF〗_exercise) and the corresponding plantar flexor power was significantly (P = 0.0046) higher at HEP56.

Soleus muscle tissue oxygenation

Soleus muscle TOI was not significantly different during the IIET at HEP56 compared to BDC (group: $P = 0.65$). In response to the IIET, soleus muscle TOI decreased significantly (time: $P < 0.001$) from 55.8% (SD = 2.9%) at rest (TOI_{rest}) to 50.8% (SD = 5.2%) during the IIET ($TOI_{exercise}$) and returned to baseline (56.0%; SD = 2.8%) during the two minute recovery phase ($TOI_{recovery}$; Fig. 6).

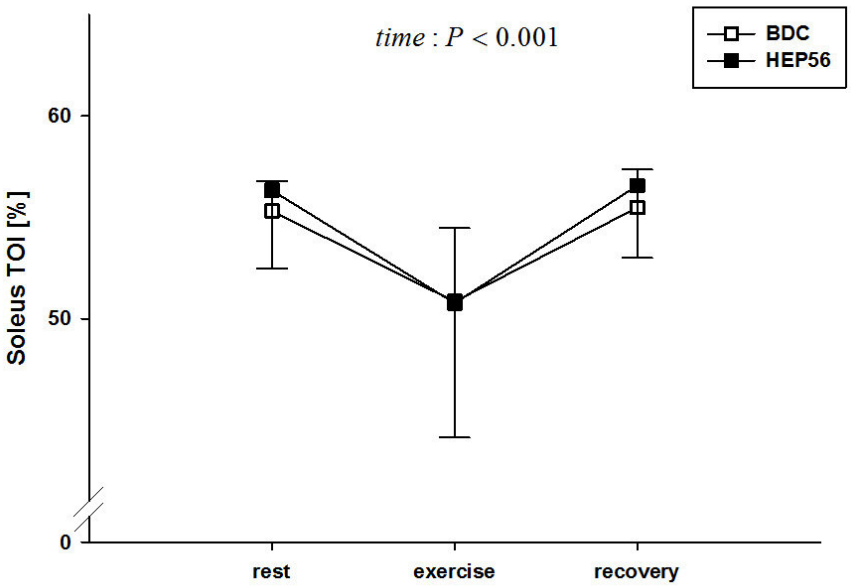

Figure 6. Soleus muscle tissue oxygenation. Soleus muscle TOI did not change significantly after the intervention (P = 0.65). In response to the IIET soleus muscle TOI decreased significantly (P < 0.001) from 55.8% (SD = 2.9%) at rest to 50.8% (SD = 5.2%) during exercise.

Electromyography

In response to the IIET, soleus muscle EMG MF decreased significantly (time: $P = 0.04$) from 111 Hz (SD = 28 Hz) during the 30% MVC stage to 101 Hz (SD = 15 Hz) during the 50% MVC stage. There is a trend (group: $P = 0.06$) that overall MFs were reduced after the intervention, however, the decrease in response to the IIET was comparable between BDC and HEP56 (group*time: $P = 0.81$). The amplitude of the EMG signal increased significantly in response to the IIET as indicated by an increased RMS throughout the experiment (time: $P < 0.001$). The increase of RMS by 109% (SD = 68%) from the 30% MVC stage to the 50% MVC stage in response to the BDC IIET appeared to be significantly more pronounced compared to the 67% (SD =57%) increase in response to the HEP56 IIET (group*time = 0.03; Fig 7).

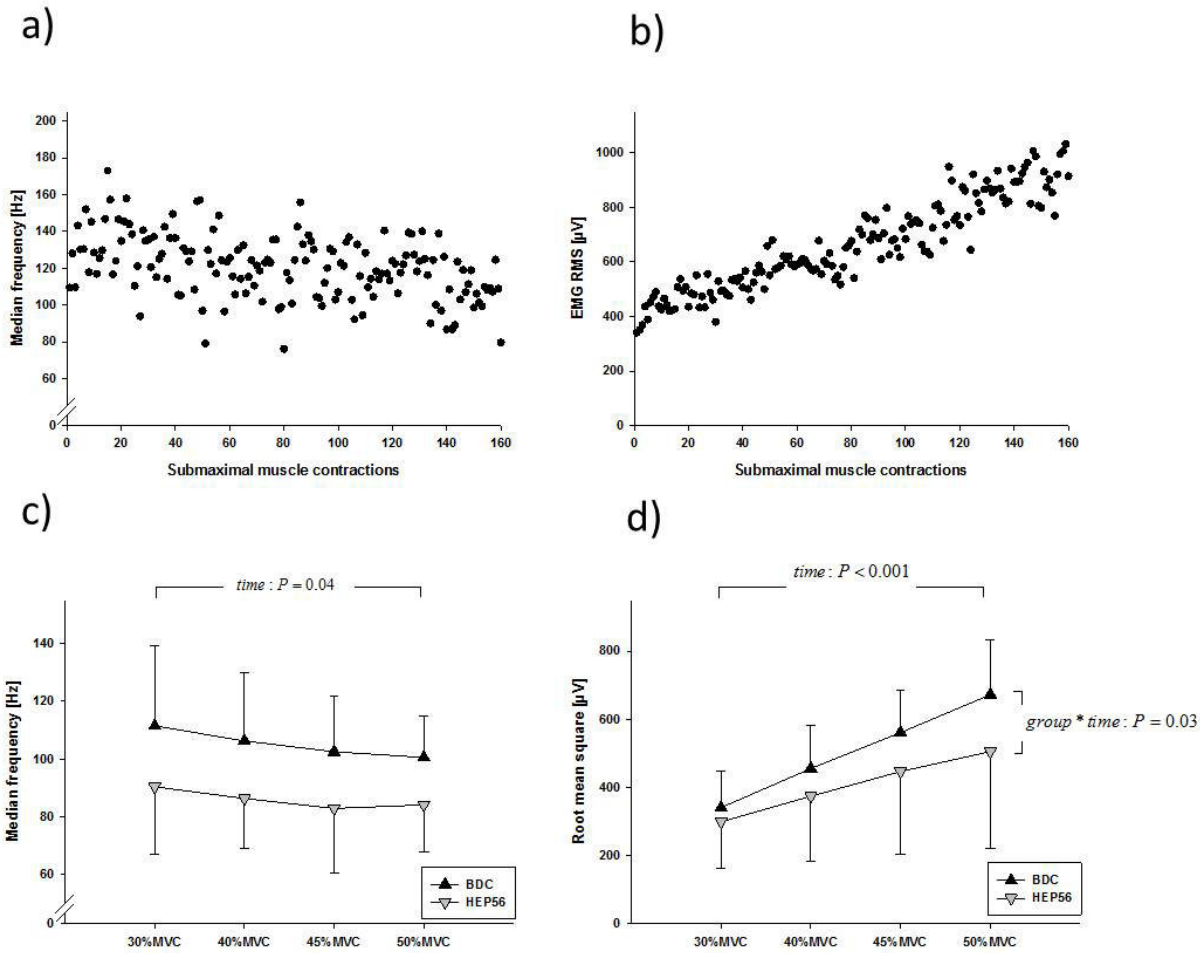

Figure 7. Soleus muscle electromyography. Shown are MF and RMS values that were calculated for each of the 160 submaximal plantar flexions. Panels (a) and (b) depict exemplary MF and RMS values for one subject. Panel (c) and (d) depict mean MF and RMS values for each stage. MF decreased significantly from stage to stage (P = 0.04) while the significant increase of RMS from stage to stage appeared to be more pronounced in response to the HEP56 IIET (P = 0.03).

Lactate concentration

There was no difference between BDC and HEP56 capillary blood lactate concentration (group: $P = 0.13$). In response to the IIET, lactate concentration increased significantly (time: $P < 0.001$) from 1.3 mmol·l^{-1} (SD = mmol·l^{-1}) at rest to 2.2 mmol·l^{-1} (SD = 0.59 mmol·l^{-1}) directly after the IIET and to 2.3 mmol·l^{-1} (SD = 0.62 mmol·l^{-1}) two minutes after the IIET.

Soleus muscle morphology

Across fibre types, fibre size (DiaMin) was significantly ($P = 0.016$) reduced by 8.5% (SD = 13%) after the intervention. Fibre-type specific analysis of DiaMin revealed only a significant reduction ($P = 0.031$) in type I fibre diameter (-11%, SD = 14%). The FCSAs of type I fibres

trended (P = 0.06) to be reduced following the intervention. Across fibre types, the mean number of CaF decreased significantly (P = 0.023) from 4.2 (SD = 1.3) to 3.6 (SD = 0.6). Capillary density (P = 0.16), capillary-to-fibre-ratio (P = 0.53), fibre type distribution (P = 0.96) and FCSA distribution (P = 0.82) remained unaltered. An overview of all biopsy data is presented in Table 1.

Table 1. Soleus muscle morphology.

	Overall	TypeI	TypeII
DiaMin pre [µm]	71±13	68±11	75±15
DiaMin post [µm]	65±8.5	61±7.7	69±7.5
P-value	*0.016	*0.031	0.21
FCSA pre [µm^2]	14592±5412	12939±4955	16244±5562
FCSA post [µm^2]	12381±3642	10202±2790	14561±3101
P-value	0.06	0.06	0.40
CaF pre	4.2±1.3	4.2±1.3	4.2±1.4
CaF post	3.6±0.6	3.6±0.6	3.5±0.6
P-value	*0.023	0.15	0.10
CD [nC/mm^2]	289±123	-	-
CD [nC/mm^2]	229±54	-	-
P-value	0.16	-	-
C:F pre [nC/nF]	2±0.7	-	-
C:F post [nC/nF]	1.9±0.7	-	-
P-value	0.53	-	-
Fibre type distribution pre [%]	-	68.4±21.9	31.6±21.9
Fibre type distribution post [%]	-	68.7±12	31.3±12
P-value	-	0.96	0.96
FCSA distribution pre [%]	-	64±22.9	36±22.9
FCSA distribution post [%]	-	62.6±13.7	37.4±13.7
P-value	-	0.82	0.82

Discussion

The main objective of the present study was to assess whether local blood supply in exercising locomotory muscles is reduced after eight weeks of local muscle unloading and if so, whether such an impaired blood supply would affect WoRPD in response to an incremental isokinetic exercise test. In contrast to our expectations and in contrast to previous observations (24), the results of this study suggest that local arterial exercise blood flow in the atrophied soleus muscle was maintained after eight weeks of muscle disuse. Furthermore, despite the slight reduction of capillaries around fibres, local tissue oxygenation, as assessed by near infrared spectroscopy did not change, nor was the intrinsic WoRPD of the plantar flexor muscle group affected by eight weeks unloading.

Muscle performance

Maximal voluntary plantar flexor torque decreased significantly after the intervention. The 23.4% loss of maximal plantar flexor torque at HEP56 is greater than what can be attributed to mere atrophy of soleus muscle fibres, which seems to be a generic finding of unloading studies (1; 25; 36). However, WoRPD, expressed as the relative power difference from MVC1-MVC5 to MVC_R throughout the IIET protocol (Fig.4c), remained unaltered. Moreover, lactate concentrations that can be used as an indication for exercise induced muscle fatigue (17) increased equally at BDC and at HEP56, reinforcing notion of an unchanged fatigability after the intervention. The concomitantly obtained EMG recordings also support this notion, as subjects did not show typical electrophysiological symptoms of increased muscle fatigue following disuse, which would be indicated by more distinct RMS increases and more pronounced MF decreases in response to exercise (19; 22). On the contrary, the amplitude of soleus muscle EMG during the submaximal muscle contractions increased less steeply after the study, which indicates that even less central drive was needed during the post HEPHAISTOS IIET (24). The trend that overall MFs appeared to be reduced after the study is in agreement with a previous study, where MFs of the vastus lateralis muscle were consistently reduced after 56 days of bed rest in response to an isometric incremental exercise test (23). The decrease of MF reflects most likely a reduction of muscle FCSA as thinner muscle fibres, if compared to thicker fibres, have a reduced conduction velocity (6) that is accompanied by a reduced initial median frequency of the EMG power spectrum (2). This endorses the morphological finding of the present study that soleus muscle fibres atrophied

after 56 HEPHAISTOS unloading. It could be argued here that the HEPHAISTOS did not entirely unload calf muscles during gait and that therefore muscle atrophy occurred without inducing any changes of fibre type distribution that can be observed in conditions associated with complete muscle silencing (Burnham et al., 1997). However, this remains speculative as it is unknown to what degree muscles need to be silenced to evoke fibre type transformations and it is also possible that 8 weeks of muscle unloading were simply too short to induce such a change. Accordingly, the absence of a fibre type transformation towards glycolytic type II fibres might have contributed to an unchanged WoRPD after the intervention.

Arterial structure and function

The structural and functional artery adaptations at rest, following 8 weeks of HEPHAISTOS unloading have been published elsewhere (33). Those data revealed that resting SFA blood flow did not change after the intervention, despite an average 12.7% (SD = 6.6%) decrease in SFA calibres at rest. This observation is in corroboration with previous unloading studies (4; 5; 12). However, the focus of the present study was on blood flow during exercise and to the best of our knowledge there are no disuse studies available to date, that investigated this. The presented data show that absolute arterial exercising blood flow remained unaltered after 56 days of HEPHAISTOS unloading. In fact, the peak SFA blood flow (BF_{rec2}) was equally increased from resting conditions to 364 $ml \cdot min^{-1}$ (SD = 139 $ml \cdot min^{-1}$) before and after the 56 days of unloading. As SFA diameters were significantly smaller at HEP56, an unchanged blood flow must have been compensated by an increased V_{mean}. At least visually, the data depicted in Fig.4b seem to corroborate that V_{mean} is consistently greater at HEP56 when compared to BDC. However, statistically, this difference failed to reach significance. Of note, the post exercise dilation of 5.8% is in accordance with the magnitude of flow mediated dilation (FMD) that was measured in the same study (33). The latter finding suggests that fatiguing, although submaximal exercise does not cause maximal vasodilation of conduit arteries to supply working muscles with blood, as previous studies showed that the FMD response does not represent maximal dilation capacity (5). Considering the above, it seems to be plausible that the unchanged WoRPD can be attributed to the unchanged arterial blood flow, since previous studies related exercise induced muscle fatigability to mainly resynthesis of phosphocreatine (PCr) that was found to be strongly linked to muscle blood flow (35). However, it needs to be stated here, that calf muscles constitute only a comparable small muscle mass and it could be argued if blood flow changes might occur when larger muscle

volumes are involved. The finding that the arterial diameter did apparently not reach its maximal dilation capacity during the IIET might thus also attributed to the relatively small volume of the working muscles.

Tissue oxygenation and blood supply

During muscle work, the tissue oxygenation index (TOI) represents a dynamic balance of oxygen consumption and oxygen delivery (7). The presented NIRS data reveal that soleus muscle tissue oxygenation was similar at BDC and HEP56. This finding suggests that blood supply to working muscles was not compromised at HEP56, as one would expect greater oxygen desaturation in poorly perfused muscles (24). The latter is reinforced by the discovery that C:F was unaffected, since the same diffusion area for oxygen was available after the study. The fact that fibre type distribution did not change after the 56-day intervention is also in agreement with the unchanged oxygen desaturation during muscle work, as oxygen consumption is dependent on oxidative capacity which in turn is thought to be largely dependent on fibre types (30). Albeit the marginal disadvantageous reduction of CaF and with regard to the atrophy of type I fibres, oxygen delivery to the working muscle might even have improved after the intervention as diffusion distances from capillaries to muscle mitochondria should have decreased. Nonetheless, the finding that blood lactate concentrations were similar between experiments, although absolute muscle work was reduced at HEP56, could indicate that the atrophied muscles relied more on glycolysis.

The ratio of blood flow (as measured during the first submaximal stage) and mechanical power (Fig.5d) suggests a surplus of arterial blood supply after the intervention. As a consequence, HEP56 TOI should be higher than BDC TOI as, with regard to the unchanged capillary-to-fibre ratio, muscle perfusion and therefore oxygen delivery should have been ‘luxurious’. Yet, TOI appeared to be similar between BDC and HEP56, suggesting that the muscle was not able to utilize the additional oxygen supplied. It could thus be that the flow that was going through the SFA did not entirely go through the capillary bed of the soleus muscle, indicating a greater arteriovenous shunt volume after the study. The present findings are somewhat different from what has been found in a previous bed rest study of the same duration (24), where the TOI and the ‘blood flow index’ as measured with NIRS under administration of indocyanine green were found to be greatly reduced. However, measurement site (soleus vs. vastus lateralis), the utilized unloading models and the applied

exercise protocols (isokinetic vs. isometric intermittent) differed between studies, making it difficult to compare the results.

Furthermore, evidence suggests that reductions of circulating blood greatly contribute to an increased exercise induced whole body muscle fatigability and a decreased O_2 uptake after periods of bed rest (10). However, in these all-out exercise tests, exercise induced muscle fatigability is not normalized for losses of strength or muscle volume. In the present study we normalized local exercise induced muscle fatigability for losses of strength and our data show that after local muscle unloading with HEPHAISTOS where muscles are greatly unloaded but not entirely silenced, blood flow to working muscles is not hindered. On the contrary, blood flow during and after exercise appears to be unaltered, suggesting a 'luxurious' conduit artery blood flow after the intervention. This might imply that peripheral vascular adaptations do not account for the disuse-induced reduction of VO_2 as seen in bed rest, at least during the first 8 weeks.

Conclusion

The presented results reveal that although maximal plantar flexor strength, soleus muscle fibre size and arterial dimensions decreased significantly, exercising blood flow and tissue oxygenation in the soleus muscle were maintained after 56 days disuse, and even increased when expressed in relative terms. Moreover, and possibly as a consequence of this, the presented data show that the soleus work related power decrease, as a measure for exercise induced muscle fatigue, following submaximal muscle work does not change after 56days of local muscle unloading with HEPHAISTOS, if normalized to maximal muscle strength. The unchanged exercise induced muscle fatigue is also reflected in the electromyographic activity of the soleus muscle where typical neuronal signs of muscle fatigue were not deteriorated. In a nutshell, the presented data suggest that the actual endurance quality of unloaded soleus muscle tissue does not change and that blood flow and oxygenation in working muscles do not constitute a limiting factor for on-going submaximal muscle work after 56 days of local muscle unloading.

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Conflicts of interest

The authors have no conflicts of interest.

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3.4 Paper 4: Whole-body vibration and arterial adaptation

Title: Vascular adaptations induced by 6 weeks WBV resistance exercise training.

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Short title: Whole body vibration and arterial adaptation

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Abstract:

Background: The impact of Whole Body Vibration (WBV) upon the cardiovascular system is receiving increasing attention. Despite numerous studies addressing the acute cardiovascular effects of WBV training, very little is known regarding long term adaptations in healthy humans.

Methods: A 6-week training study, with a 70days follow-up was designed to compare resistive exercise with or without super-imposed whole body vibrations. Arterial diameter, Intima Media Thickness and Flow Mediated Dilation (FMD) were assessed by ultrasonography in the superficial femoral artery (SFA), the brachial (BA) and the carotid arteries (CA).

Results: SFA resting diameter was increased from 6.22mm (SD = 0.69mm) at baseline to 6.52mm (SD = 0.74mm) at the end of the training period (P = 0.03) with no difference between groups (P = 0.48). Arterial wall thickness was significantly reduced by 4.3% (SD = 11%) in the CA only (P = 0.04). FMD was not affected by any of the interventions and in any of the investigated arteries.

Conclusion: To the best of our knowledge, this has been the first study to show that the superposition of vibration upon conventional resistance exercise does not have a specific effect upon long term vascular adaptation in asymptomatic humans. Our findings seem to be at variance with findings observed in a bed rest setting. One possible explanation could be that the independently saturable effects of flow-mediated *vs.* acceleration-related endothelial shear stresses on arterial structure and function differ between ambulatory and bed rest conditions.

Key words: Resistive Vibration Exercise, Arterial Structure, Arterial Function, Gravitational Induced Shear Stress, Flow Mediated Shear Stress.

Introduction

It is generally accepted that vessels, including large arteries, can adapt their structure as well as their functioning in response to alterations in their environment. As such, chronic disuse (*e.g.* spinal cord injury or bed rest) induces reduction in arterial diameter (7; 8; 10) and alterations in the ability to dilate (7; 8; 10). On the other hand it has been shown that exercises such as running, cycling, or walking can improve arterial function and structure and are through these direct vascular conditioning effects able to modify cardiovascular risk (35). Of note, regular physical exercise is associated with a reduction of vascular events (6; 13), which highlights the importance of the matter under discussion.

It is mostly held that arterial adaptations occur in response to changes in shear rate acting on the endothelial layer (17; 38). Since shear rate is dependent on flow velocity and vessel cross sectional area, muscle work influences internal vessel shear rate directly through increases of blood flow.

Whole body vibration (WBV) is a novel exercise modality that is receiving increasing attention (24). Among many other things, WBV is affecting the cardiovascular system acutely, leading to an increase in blood flow velocity and tissue perfusion (14; 19) that is parametrically depending upon the frequency and amplitude of vibration (18). The increased blood flow is thought to be in direct proportion to the enhanced oxygen demand by the working musculature (26). Initially, however, tissue oxygenation of the calf muscles appears to be increased during WBV, indicative of a 'luxury' perfusion for the acutely working muscle (28). As one possibility it has been suggested that this effect is driven by endothelial shear stress (27). The latter is quite likely to increase when the vibration is in line with the vessel axis (40).

Very little, however, is known regarding the long-term effects of vibration upon vascular adaptive processes. In a bed rest setting, Bleeker et al. (8) found that WBV in combination with conventional resistive exercise maintained the diameter in the leg conduit arteries of the exercise group and thus attenuated the decrease that was observed in a control group during 56 days of bed rest. However, there was no group that performed WBV only, or resistive exercise only, and it was therefore impossible to determine the specific effects of vibration.

That question was addressed in a follow-up study that included an additional 3rd group that performed resistive exercise without vibration (5). It was found that vibration did indeed have a beneficial effect upon the conduit arteries above that was not achieved by resistive exercise alone (39).

The question arising from these studies is, however, whether vibration would have a specific effect upon long-term vascular adaption in people who are not subjected to bed rest. As, to the best of our knowledge, there is yet no study available that addressed this question, and based upon the theoretical framework outlined above, we hypothesized that superposition of vibration upon conventional resistive exercise would enhance the increase in diameter and in vascular dilation capacity within the setting of a training study.

The aim of the present study was, therefore, to examine the effects of 6 weeks of resistive exercise training with and without WBV exposure upon structure and function of the human vasculature, as well as the time course of any such effect. As a tertiary study aim, we sought to determine the retention of training-induced changes in vascular structure and function following 70 days after cessation of the training program. Given the training-regime involved the leg musculature, the focus of our attention was the superficial femoral artery (SFA). The brachial artery (BA) and the carotid artery (CA) were additionally studied to assess any systemic effects of the training regime.

Methods

Study Design and Subjects

The effects of Vibration Exercise study (EVE-study) was designed as a stratified, randomized two-group parallel design. Twenty-six healthy men (26±4 years) were recruited as participants. Two matched groups with regards to their maximum vertical jump height as an indicator of neuromuscular fitness (30) were formed. A coin was then tossed to determine which group would perform either resistive vibration exercise (RVE) or resistive exercise (RE) only. Table 1 presents the anthropometric data at baseline. All subjects had been examined by a medical doctor before study inclusion. Exclusion criteria were: diabetes; any known cardiovascular disease or abnormality; smoking; participation in strength training during the past 6 months; or any regular medication. Written informed consent was obtained

from all subjects before commencement of the study. The EVE study protocol was approved by the Ethics Committee of the Northern Rhine medical association (Ärztekammer Nordrhein) in Duesseldorf (a rural suburb of Cologne).

Table 1. Subject characteristics

	RVE-group (n=13)	RE-group (n=13)	p-value
Age (yrs)	24,31 (±3,28)	23,38 (±1,39)	0.52
Body mass (kg)	74,7 (±6,94)	75 (±4,67)	0.08
Height (m)	1,79 (±0,05)	1,79 (±0,05)	0.31
BMI	23,46 (±2,1)	23,38 (±1,4)	0.11
systolic blood pressure (mmHg)	121(±4)	127(±8)	0.15
diastolic blood pressure (mmHg)	71(±6)	72(±9)	0.89
Heart rate (beats/min)	57(±8)	55(±9)	0.70
Vertical jump height (cm)	41.7 (±2.2)	42.2 (±4.6)	0.97

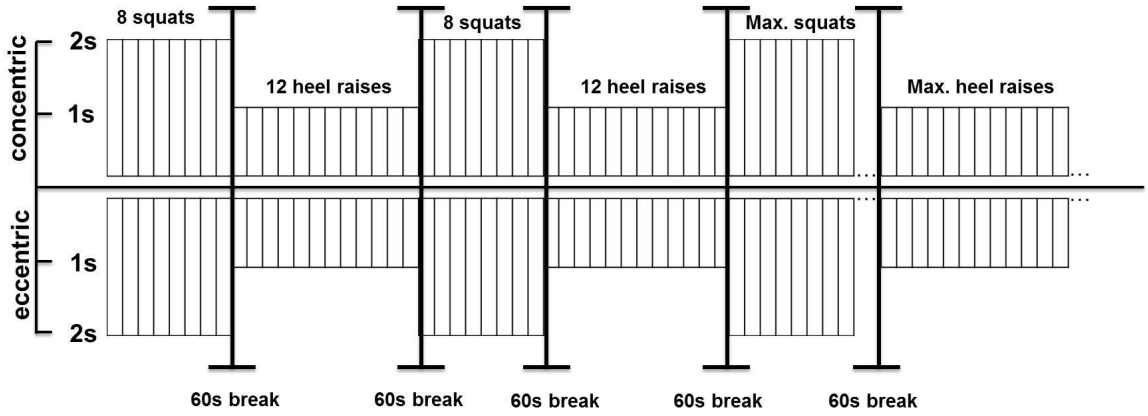
Procedures

Training protocol

Exercises were performed using a guided barbell (Hoist fitness, San Diego, USA) and a side alternating vibration plate (Galileo Fitness, Novotec, Pforzheim, Germany). All participants were familiarized with the training and equipment before the first training session. Subsequently, the individual training load was determined at 80 % of the 1-Repetition Maximum (1RM; 80 % of the 1RM equals 8 repetitions of squats; squats were used as a reference to determine the individual training load) using the method described by Baechle and Earle (3). Briefly, the subjects were loaded with an estimated weight and were then asked to complete as many repetitions as possible. The initial training load was then adjusted to i) a higher load if the subject completed more than 8 repetitions of squats or ii) to a lower load if the subject completed fewer than 8 repetitions of squats. During the first two weeks of the training intervention, two training sessions per week were completed. From the third week until intervention end, training was performed 3 times per week. As a warm up, 2 sets of heel raises and squats, in alternating order, were performed using the barbell (~ 15 kg) without additional weights. A metronome was used as time emitter. The amplitude for the vibration

was set to 6 mm (peak to base displacement) by the position of the feet on the plate. For the last set, the subjects should perform as many repetitions as possible. The individual load was recalculated after every training session applying the 1RM-method (3) and using the last set of squats as a reference. The vibration plate was centred under the guided barbell, to allow optimal exercise performance. The RE group performed the exercises while standing on the same vibration plate, but without vibration stimulus. Each training session was supervised by an exercise scientist and took approximately 9 minutes. Blood pressure and heart rate were measured during each break. The protocol for the training sessions, as well as the exercise progression scheme are depicted in Fig. 1.

a)



b)

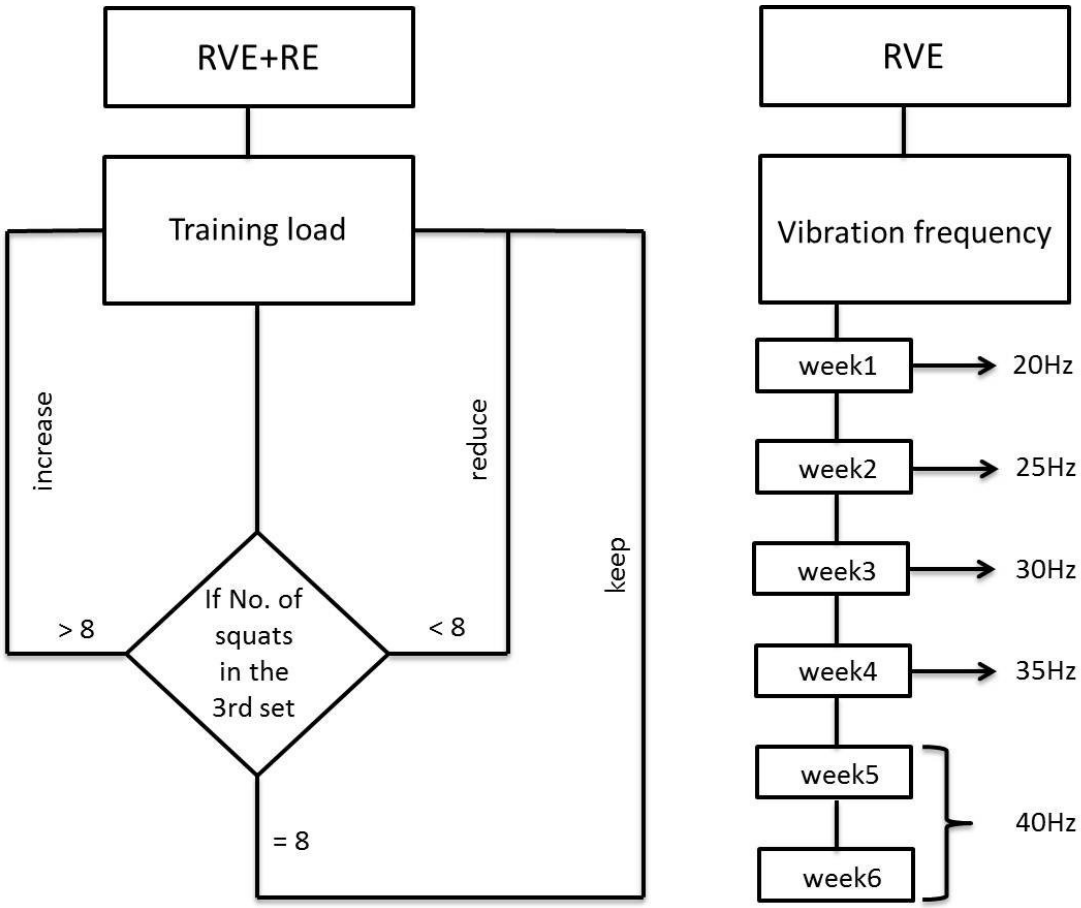


Figure 1. Exercise modalities. a) Composition of one training session after warm up. The subjects accomplished 3 sets per exercise, with a 60s break in-between the sets. In the last set of each exercise the subjects were asked to perform as many repetitions as possible. b) The individual load was recalculated after every training session using the 1RM-method with the last set of squats as a reference. The vibration stimulus for subjects in the RVE group was weekly increased with 5Hz from 20 Hz to maximally 40Hz during week 5 and 6.

Ultrasound measurements

Arterial diameters, intima media thickness (IMT), and flow mediated dilation (FMD) were examined at baseline, after 1 week of training, after 3 weeks of training, after 6 weeks of training and 3 months after the last training session (respectively, BDC, EVE7, EVE21, EVE42 and follow-up). IMT was measured in the SFA and in the CA, while resting diameters, blood cell velocity and FMD were measured in the SFA and in the BA. Blood cell velocity and resting diameter measurements of the BA were performed using an echo Doppler device (Mylab25, esaote, Firenze, Italy) with a 12-18 MHz broadband linear transducer. Blood cell velocity and resting diameter measurements of the SFA were performed with a 7.5-12 MHz broadband linear transducer. Anatomical landmarks such as the upper patella edge (for the SFA) and the radius epiphysis (for the BA) were recorded for all arteries to ascertain reproducibility of probe placement. Continuous measurement of velocity and diameter were performed using duplex ultrasound. For resting diameter measurements, videos with duration ≥ 1min were recorded for offline analysis. For FMD assessment of SFA and BA, a cuff was placed distal to the probe that was inflated to 300 mmHg for 5 min. 10s prior to cuff deflation video recording was started, and the FMD response was recorded for 5 minutes after cuff deflation. All videos were recorded on an external computer, using the analogue output of the device with a video grabbing system (GrabsterAV 450MX, Terratec, Nettetal, Germany) and an analogue to digital transformation software (MAGIX, Terratec, Nettetal, Germany). All measurements were performed at the same time of the day to avoid circadian variation. Prior to the measurements, subjects rested in a darkened room for at least 20 minutes in supine posture. Subjects fastened prior to the measurements for ≥ 8h and refrained from caffeine, alcohol and exercise for ≥ 8h before the measurement.

Data processing

Intima Media Thickness

The IMT was determined by the IMT software tool (esaote, QIMT, for MyLab25). The IMT analysis tool processes the radio frequency signal (RF-signal) from the ultrasound device in real time. IMT videos were recorded for ≥5 heart cycles, using a 7.5-12 MHz broadband transducer placed parallel to the assessed artery. The region of interest (ROI) for IMT measurements was placed at the region of the artery with the highest image quality. IMT video analysis was performed using a video sequence of ≥ 5 heart cycles. The IMT videos

were analysed offline and the value with the lowest standard deviation (≤ 20µm) was taken as IMT. Blood pressure was measured at 5 time points before the 1st measurement and during each cuff inflation/deflation period, using an electronic sphygmomanometer (medicus pc, boso, Jungingen). Heart rate was measured continuously using the internal 3-lead ECG of the ultrasound device.

Figure 2. Off-line video analysis. Diameters within the ROI were measured continuously using the "Vasculometer"- edge detection (9) software and a sampling frequency of 25Hz.

Diameters and Flow Mediated Dilation

All videos were analysed off-line. Duplex video analysis was performed using a custom produced edge detection and wall tracking software (Vasculometer 1.2, (9). The signal from the wall tracking software was processed with MATLAB (Mathworks, Natick, MA, USA, Fig.2), using a moving average filter with a span of 500 frames. The median of all processed values before cuff deflation was taken as resting diameter. The highest value of the filtered signal was identified and used as peak diameter after cuff release. The FMD response was then expressed as the relative increase in diameter after cuff deflation (see Fig. 3).

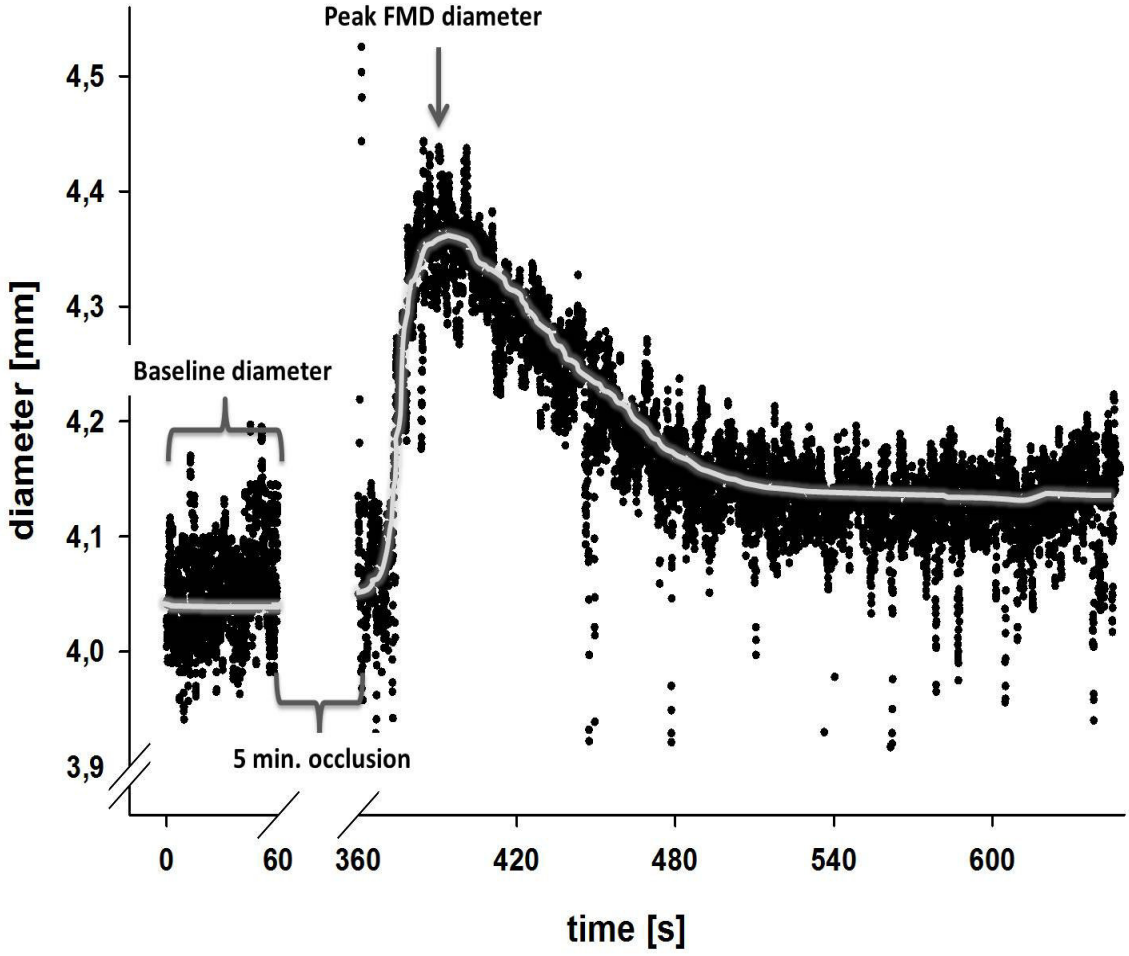


Figure 3. Signal Processing for FMD analysis. The dark signal depicts the raw signal of the brachial artery diameter of one subject as measured with the automated wall detection software. The white line plots the signal, filtered with a moving average filter (span: 500 frames). The diameter was not recorded during the occlusion. All measurements were performed with a sampling frequency of 25 Hz.

Statistical analysis

Statistical analyses were performed using STATISTICA 8.0 for Windows (Statsoft, Tulsa, Oklahoma, USA, 1984-2008). A Repeated measures ANOVA was performed with time (five different points) and group (RVE *vs.* RE) as main factors, as well as an interaction between main factors. A repeated measures ANCOVA was performed to assess the effect of training load progression as a covariate on SFA baseline diameters. Values are given as means±SD. There were 10 missing values out of 156 in the RE group and 12 out of 156 in the RVE group. Those values were linearly interpolated using adjacent data. Tukey's Test was used for post

hoc testing. Differences regarding the anthropometric characteristics at baseline were assessed performing a non-paired t-test.

Results

The subject's anthropometric characteristics, as well as maximum countermovement jump height were comparable between groups (see Table 1), and anthropometric characteristics were unchanged during the course of the study. Due to medical reasons two of the initially 15 starting RVE-subjects were not able to complete the intervention. One subject had to quit the study after 2 weeks because an acute back injury induced by the training, the other subject dropped out after the 4th intervention week because of exercise related headache. It was not possible for the subjects of the RVE group to reach maximal plantarflexion during the heel raise exercise, however, this "handicap" was not noted for the subjects of the RE group.

Training progression

From the beginning of the intervention both groups showed an almost linear increase of the training loads (see Fig. 4). Compared to the first training session, the increase of training loads during the 6 weeks intervention reached 46.9% (SD = 18.9%) in the RVE group, which was less than the increase of training loads by 59.8% (SD = 17.3%) observed in the RE group (time*intervention: $P < 0.001$).

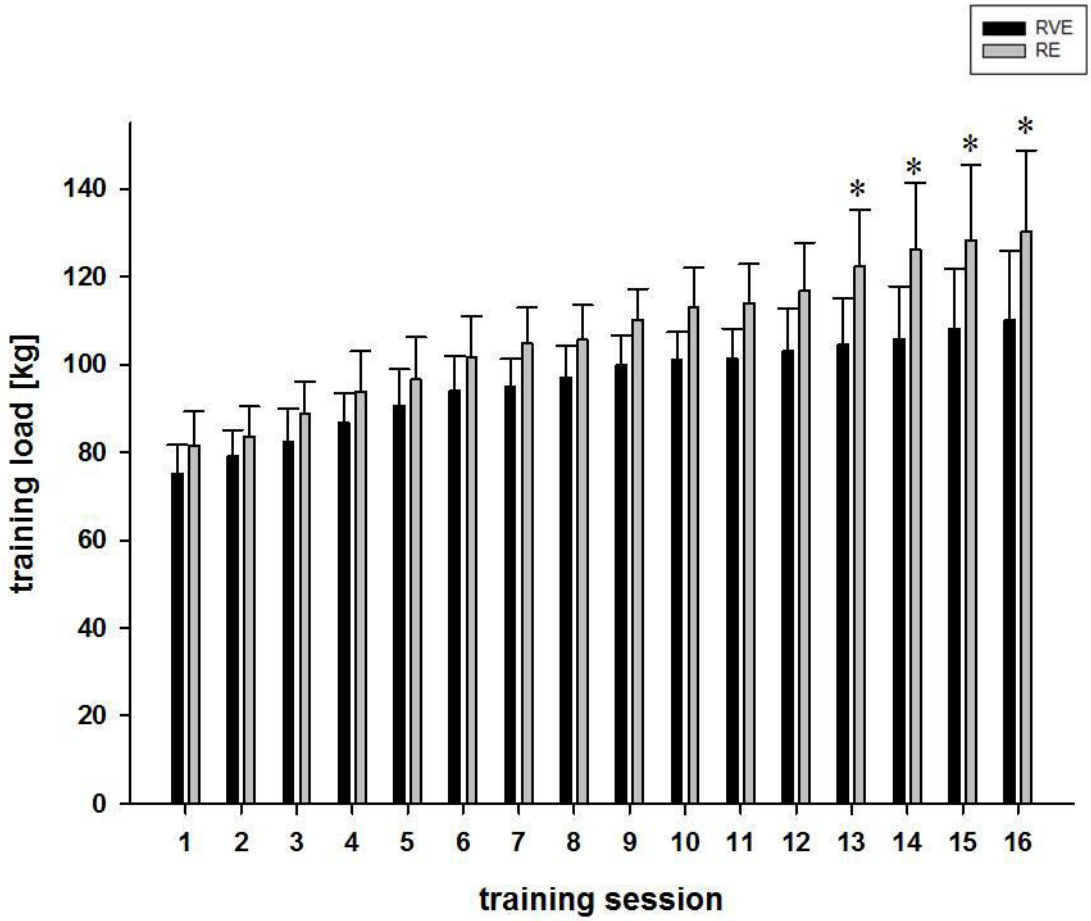


Figure 4. Increase of training loads. The individual load was recalculated after every training session using the 1RM-method with the last set of squats as a reference. The mean training load of the RE group was higher throughout the whole intervention. The difference between the groups reached significance after the 13th training session (time: P < 0.001; time*intervention: P < 0.001).

Arterial Diameter

All structural and functional parameters are illustrated in Fig. 5. Compared to baseline data collection (BDC), SFA resting diameter increased in both groups by 7.2% from the 3rd training week (EVE21) onwards ($P < 0.001$), after which time there was no further increase observed ($P = 0.58$; see Fig. 5a). There was a tendency ($P = 0.06$) that the diameter was still increased after 70 days following training, comparing follow up and BDC diameters (see Fig. 6). The resting diameter of the BA was not affected by any of the interventions, however, a tendency (time: $P = 0.06$) reveals a slight systemic adaptation of the BA over time for both groups (see Fig. 5b). We did not detect any significant difference between the two interventions regarding the time course and the magnitude of the diameter adaptations for both SFA (time*intervention: $P = 0.96$) and BA (time*intervention: $P = 0.20$).

Intima Media Thickness

No effect of time ($P = 0.42$) or intervention ($P = 0.11$) was observed for the IMT of the SFA (see Fig. 5c). Intima Media Thickness of the CA was significantly lower (- 4.2%) after the intervention but no difference was observed between the two groups (time: $P = 0.04$; time*intervention: $P = 0.11$; see Fig. 5d).

Flow Mediated Dilation

No effects of time or intervention were observed for the FMD response of the SFA (time: $P = 0.45$; time*intervention: $P = 0.60$; see Fig. 5e) or the BA (time: $P = 0.27$; time*intervention: $P = 0.99$; see Fig. 5f). Furthermore, the time to peak FMD for both the SFA (time: $P = 0.46$; time*intervention: $P = 0.25$) and the BA remained unaltered for both groups throughout the experiment (time: $P = 0.44$; time*intervention: $P = 0.15$).

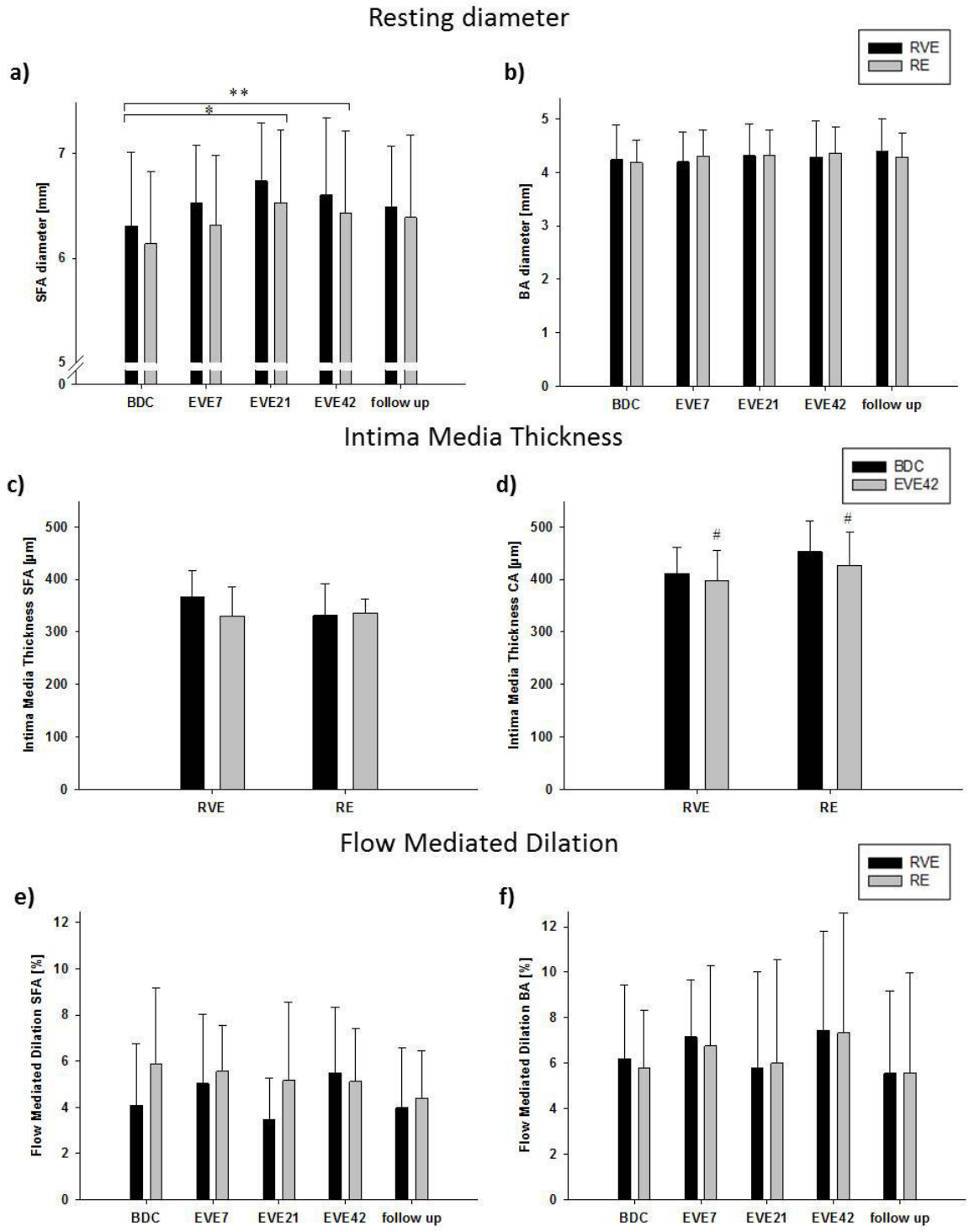


Figure 5. Arterial parameters. Panel a depicts the time course of SFA resting diameter throughout the study for both RE and RVE. No significant differences existed between the responses of both groups. Across groups, SFA diameter significantly increased from EVE21 onwards by 7.2% (P = 0.003, * P < 0.001). Panels b and c show, respectively, that there were no changes in BA diameter and SFA IMT. Panel d shows that CA IMT was significantly reduced after the intervention (# P = 0.04). Panels e and f show that the FMD remained unaffected for both SFA and BA.**

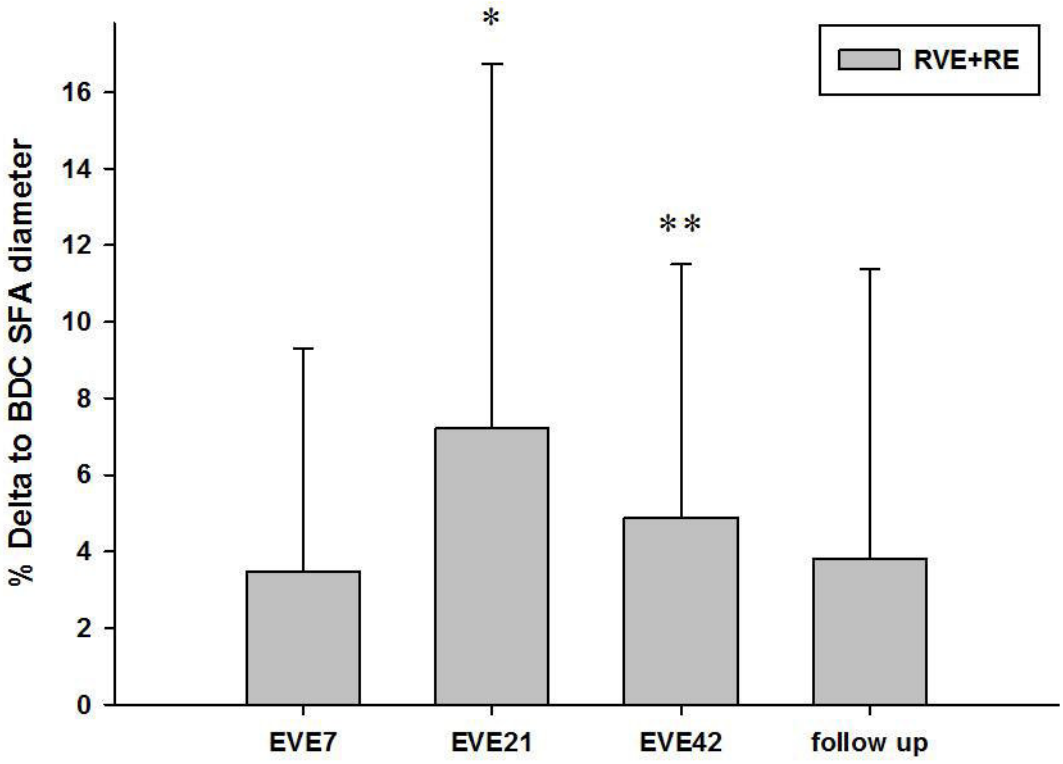


Figure 6. SFA resting diameter adaptation. Relative values of the SFA resting diameter increases, using the pooled data of both intervention groups. Resting diameters are significantly increased for EVE21 (* P < 0.001) and EVE42 (P = 0.003) compared to the BDC values. There is a tendency that the diameters are still increased 70 days after the intervention, comparing follow up and BDC values (P = 0.06).**

Discussion

The main aim of the present study was to investigate the specific effects that whole body vibration might have in a training setting upon structure and function of conduit arteries in healthy ambulatory subjects. The results suggest that RE training and RVE training equally lead to increases in resting diameter of the SFA. This finding is line with the existing literature in that it supports the notion of training-induced enlargement of arterial diameter (22; 29; 35). However, in contrast to our expectations, there was no difference between the two intervention groups in any of the parameters tested in the present study. Moreover, no effect by the training intervention was elicited upon flow mediated dilation or intima media thickness. Both of these observations are in stark contrast to findings in a bed-rest setting (8; 39).

Changes of SFA resting diameter

Our main hypothesis was based upon the view that i) endothelial shear stress is a main driver in adaptation of resting diameter in the conduit arteries, and ii) that vibration exercise will enhance endothelial shear stress. It is our opinion that the current data, although collected meticulously and with great care, do not provide strong enough evidence to put the first assumption into question because of the bulk of literature in their support (4; 16; 37). In many biological systems, however, the frequency composition of a controlling signal is of great importance, and it seems that considering the frequency composition of shear stress imposed by arterial (= pulsatile) flow could be relevant, too. In light of this consideration the obvious conclusion from the present study would be that it could be flow-mediated, rather than acceleration-induced shear stress that matters for the adjustment of resting diameter. On the other hand, this explanation would be at variance with the aforementioned findings in the bed-rest setting (8; 39). Importantly, there is no substantial metabolic demand upon the immobilized leg musculature, and flow-mediated shear stresses must therefore be expected to be low. In this 'bradytrophic' situation, the provision of resistive exercise might engender increased blood flow in the absence of any substantial acceleration-induced shear strains in the conduit arteries. Of note, walking and running is associated with vertical accelerations of up to 10 g (15), meaning that the habitual activity in an ambulatory setting is indeed likely to provide acceleration-related endothelial shear stresses. One way of reconciling findings from this study with those from the aforementioned bed rest studies, therefore, would be in assuming that acceleration-induced shear stress does have an effect upon resting diameter that is independent of flow-mediated shear stress, and that this effect saturates under ambulatory conditions with habitual activities. Although the training loads differed significantly between RVE- and RE group for the last four training sessions, training load progression did not have any measurable effect on resting SFA diameters, as yielded by ANCOVA ($P = 0.17$, data not shown).

Time course of adaptive changes and their retention

Previous studies showed that functional adaptations occur rapidly after the onset of an exercise intervention and precede structural adaptations (12; 36). However, we did not detect any interplay between functional and structural adaptations, regarding their time course. An interesting finding of the present study is in relation to the time-course of vascular adaptation: the change in SFA resting diameter has reached its maximum after the 3rd training week

(EVE21) and did not further increase during the subsequent 3 training weeks. The steady state in luminal expansion of the SFA could not be overcome by the progressive increase in training loads by increasing weight and vibration frequency (the latter RVE only) during the study. One way of explanation would be that a functional state had been achieved at EVE21 that did not necessitate any further increase in flow capacity to accommodate the increase in exercise-related energy expenditure. In this context it is useful to consider that the flow capacity of skeletal muscle is by far greater than could be covered by the cardiac output (1).

Investigations of the retention of training effects provide helpful information about the preventive quality of a training regime. To date, only few studies investigated the retention of training interventions regarding vascular adaptation effects. We found that the BDC resting diameter of the SFA was not statistically different compared to the diameter measured at the follow up session 70 days after intervention end. However the difference between the BDC resting diameter and the diameter measured 90 days after the intervention failed to reach statistical significance only by a small amount ($P = 0.06$), revealing the possibility that some structural adaptation might have been maintained beyond the end of the intervention and just faded away before the follow-up testing (see Fig. 6).

Intima Media Thickness

Intima media thickness, as measured with B-mode ultrasound, provides an index of sub-intimal thickening. IMT of the carotid artery for example is commonly used as a surrogate marker for preclinical atherosclerosis and is strongly related to cardiovascular risk factors and diseases (32). Previous studies reported no or only a modest impact of exercise interventions on carotid artery IMT (23; 31). However, in the present study both intervention groups showed a significantly reduced IMT of the CA. Thijssen et al. (32) recently concluded that high exercise intensities or high exercise volumes are required to affect carotid artery IMT. Indeed, the present study does not satisfy the latter aspect because the subjects exercised only for approx. 30min/week, but since they trained with very heavy loads (80% MVC) one could commonly regard the present training regime as “intense”. Furthermore, the effects of exercise interventions on the IMT of peripheral arteries that supply the exercising muscles are thought to be more pronounced than in the CA (20). Nonetheless, there were only significant changes in the CA but not in the SFA in the present study. One explanation for this finding could be that the mean SFA IMT of all subjects (348µm, SD = 57µm) was already too low to

detect further decrease adaptations, whereas the mean CA IMT (432µm, SD = 57µm) still had some "buffer" to further adapt downwards.

Flow Mediated Dilation

Physical exercise is thought to improve FMD, a measure of endothelial function. In this context, blood flow-induced shear stress acting on the endothelial layer seems to be the main driver. Since different exercise regimen lead to different blood flow patterns (33) and different exercise intensities lead to acute changes in the bioavailability of Nitric Oxide (NO) (11), previous training interventions showed heterogeneous findings regarding their impact upon vascular function. Goto et al. (11), for instance, showed in their study that only training at moderate intensities (50% VO_2-Max) was able to affect FMD. This would explain the findings from the present study, whereas FMD was not affected by any of the two interventions. Furthermore, the present results confirm earlier findings in healthy subjects (23; 34) and suggest that arterial function is more prone to enhance in patients (2; 21) than in healthy individuals. However, our findings remain discordant with the findings observed in a bed rest setting that showed that only RVE was able to attenuate the immobilization induced increase of FMD (8; 39), while RE failed to impact FMD. Though, the mechanisms responsible for the altered hemodynamic situation during WBV are currently unclear. Both an increased metabolic demand of the working muscles during WBV (25), as well as the arterial wall accelerated with the other leg tissues around the "inert" blood column might constitute a crosstalk of shear stress- trigger signals. A separated analysis of the arterial blood flow during passive vibration, during RE alone and during RVE, admittedly a very challenging approach, would provide helpful information to complete our picture about the hemodynamic situation during WBV.

Conclusion

In conclusion, six weeks of resistive training for 3 times per week led to significant adaptations of the SFA diameter regardless of whether it was combined with whole body vibration or not. No intervention had an effect on arterial function. We did not observe local effects but we observed systemic effects concerning changes in wall thickness. These findings seem to be at variance with findings in bed rest. Of note, the subjects being investigated in the present study were healthy subjects. A similar study design applied to a diseased population with poor vascular structure and function might be more in line with the findings observed

under bed rest conditions. One possible explanation could be that the independently saturable effects of flow-mediated *vs.* acceleration-related endothelial shear stresses on arterial structure and function differ between ambulatory and bed rest conditions. However, RVE training as conducted in the present study is highly demanding and exhaustive for the subjects and exercise parameters would therefore have to be tailored for people in a diseased state.

Perspective

As WBV is an exercise modality that is gaining more and more popularity across all kinds of gyms, we wanted to investigate its effects upon arterial structure and function in healthy ambulatory subjects. Our data suggest that resistive exercise leads to both an increase of the resting diameter of the femoral artery and a decrease of the carotid artery wall thickness, and that these effects are not enhanced by super-imposed vibration. The underlying acute hemodynamic situation during bouts of vibration exercise, however, needs to be further investigated to better understand the influence of different blood flow patterns and to explore the impact of gravity- driven, acceleration- related shear stress, acting on the endothelial layer.

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4 Chapter Four – Primary findings and conclusion

It were the primary aims of the present thesis to investigate the specific role of gravity impacts as a mechanical signal to induce arterial adaptations and to find out if prolonged muscle disuse leads to functional impairments of local blood flow. Thus far, research addressing arterial adaptations has only considered intrinsic hemodynamic signals that are applied by means of blood acting on the arterial wall. However, it seems to be inappropriate to omit the existence of gravity impacts in this consideration, given upright human locomotion in our gravitational environment. It could be shown in previous studies that if the human body or parts of the human body are gravitationally unloaded, arteries degenerate dramatically (1-4; 7). Thus, the applied unloading models have all in common that muscle work reduction is always accompanied by a great reduction of gravity impacts, and it has so far not been possible to attribute arterial degeneration to either the lack of muscle work or to the absence of gravity impacts. In order to investigate the arterial conditioning effect of gravity impacts independently from muscle work, we utilized the HEPHAISTOS unloading orthosis. The results of the HEPHAISTOS study (HEP-study) disclose that muscle work is needed to maintain arterial calibers as arterial downsizing within the scope of the HEPHAISTOS intervention was comparable to what has been found in bed rest, limb suspension or spinal cord injury (1-4; 6-8). Nonetheless, the observation that wall-to-lumen ratio and endothelial function in terms of flow mediated dilation (FMD) were unaltered seems to be at variance with previous disuse studies where wall-to-lumen ratio and flow mediated dilation were reported to increase (2; 5; 7; 8). Hence, the present findings suggest that habitual gravity impacts constitute an important stimulus for these parameters.

The results of the Effects of Vibration Exercise study (EVE-study), however, reveal that high frequent vibrations leading to ‘artificial’ gravity impacts do not have an additional effect on arterial adaptation if superimposed to resistive exercise training. It was observed that arterial calibers increased equally in response to strength training, no matter if vibrations were superimposed or not. These findings are not in accordance with observations made in bed rest (2; 7; 8) where a vibration-specific arterial conditioning effect was found leading to both attenuations of arterial caliber decreases and wall thickening as well as to maintenance of endothelial function. As the subjects of the EVE study were ambulatory, it could well be that the vibration effect was covered since the ‘demand’ for gravity-induced stress might already

have been saturated through habitual gravity impacts. In conclusion, the primary findings of these two studies related to arterial adaptations highlight the importance of muscle work-driven blood flow. Moreover, it seems that gravity-induced impact loading contributes to mechanical signaling and may have a direct conditioning effect for arterial structure and function, which has not been considered before.

In addition, it could be shown in the present thesis that functional exercise hyperemia is not impaired after prolonged local muscle disuse although arterial dimensions, muscle size and muscle power decreased significantly and that likely, as a consequence, exercise tolerance can be maintained.

Taken together, the results of the present thesis underline the importance to consider mechanical forces in muscle and vascular physiology in conditions associated with exercise and muscle disuse.

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5 Chapter Five – Addendum

5.1 Zusammenfassung in deutscher Sprache

Hintergrund: Es ist bekannt, dass Muskelentlastung oder (Ausdauer-) Training die Struktur und Funktion von Gefäßen direkt beeinflussen und unter anderem durch diesen Effekt direkten Einfluss auf kardiovaskuläre Risikofaktoren nehmen können. Die zugrundeliegenden Mechanismen sind bisher allerdings nicht gänzlich bekannt. Klar ist, dass insbesondere Endothelzellen und die glatte Muskulatur der Arterien auf mechanische Belastung reagieren. Als mögliche Ursache für diese mechanische Belastung wurden bisher ausschließlich intrinsische hämodynamische Größen in Betracht gezogen. Dieser Ansatz erscheint allerdings nicht komplett da es in Anbetracht unseres Schwerkraftumfeldes auf der Erde zu einer erheblichen Anzahl von Aufschlägen kommen muss, durch die (Körper-) Masse beschleunigt wird. Ein wesentliches Ziel der vorliegenden Arbeit besteht daher darin herauszufinden ob diese Art der Massebeschleunigungen, während Muskelentlastung und Training, für die Adaptation von Arterien von Bedeutung sind. Darüber hinaus stellt die Beziehung zwischen arteriellem Blutfluss, Muskelperfusion und dynamischer Muskelermüdung nach längerer Muskelentlastung ein zentrales Thema dieser Arbeit dar.

Methoden: Es wurden zwei klinische Interventionsstudien durchgeführt. 11 gesunde männliche Probanden nahmen an der HEP-Studie teil. Im Rahmen dieser Studie wurde die Wadenmuskulatur der Probanden für 56 Tage mit einer neuartigen Orthese (HEPHAISTOS) entlastet, ohne dass dabei der Einfluss von Schwerkraft-induzierten Massebeschleunigungen verändert wurde. Die EVE-Studie wurde durchgeführt um die Effekte von konventionellem Krafttraining und zusätzlichen Ganzkörpervibrationen auf die Anpassung von Arterien zu untersuchen. Dabei nahmen 26 gesunde männliche Probanden teil, die entweder ein 6-wöchiges konventionelles Krafttraining oder Krafttraining mit zusätzlichen Vibrationen absolvierten. Zu den zentralen Messmethoden gehörten Ultraschallmessungen, die in beiden Studien durchgeführt wurden um sowohl strukturelle als auch funktionelle Arterienparameter zu erfassen. Darüber hinaus wurden im Rahmen der HEP-Studie Muskelbiopsien entnommen um morphologische Veränderungen der entlasteten Muskulatur zu erfassen. Außerdem wurden Nah-Infrarot spektroskopische Messungen durchgeführt um die Sauerstoffsättigung der Muskulatur während Muskelarbeit, vor und nach der Entlastung zu bestimmen.

Ergebnisse: Die Muskelentlastung mit der HEPHAISTOS-Orthese führte zu einer signifikanten Abnahme des Lumens der Femoralarterie, wobei Gefäßwand und Endothelfunktion unverändert blieben. Obwohl sich die Größe der Femoralarterie und die Größe des entlasteten Soleus-Muskels signifikant verringerten, hatte dies keinen Einfluss auf Blutfluss, Sauerstoffsättigung und Ermüdbarkeit. Durch die Trainingsintervention in der EVE-Studie kam es zu einer signifikanten Vergrößerung des Lumens der Femoralarterie und zu einer Abnahme der Gefäßwanddicke der Arteria Carotis, wobei die applizierten Ganzkörpervibrationen keinen zusätzlichen Effekt hatten.

Schlussfolgerung: Beide Studien zeigen, dass Muskelarbeit und damit intrinsische hämodynamische Kräfte einen wesentlichen Einfluss auf strukturelle und funktionelle Arterienanpassung haben. Dennoch scheinen Schwerkraft-induzierte Massebeschleunigungen einen Effekt auf Anpassungen der Arterienwanddicke und der Endothelfunktion zu haben, womit sie einen direkten Einfluss auf Parameter für kardiovaskuläres Risiko hätten. Außerdem wurde gezeigt, dass sich Blutfluss und Sauerstoffsättigung während Muskelarbeit, nach längerer Muskelentlastung nicht ändern, was wiederum die unveränderte dynamische Muskelermüdung erklären würde.

5.2 Original manuscripts

The specific role of gravitational accelerations for arterial adaptations

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The specific role of gravitational accelerations for arterial adaptations

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Weber T, Ducos M, Mulder E, Herrera F, Brüggemann G, Bloch W, Rittweger J. The specific role of gravitational accelerations for arterial adaptations. *J Appl Physiol* 114: 387–393, 2013. First published December 6, 2012; doi:10.1152/japplphysiol.01117.2012.—It is mostly agreed that arterial adaptations occur, among others, in response to changes in mechanical stimuli. Models like bed rest, spinal cord injury, or limb suspension have been applied to study vascular adaptations to unloading in humans. However, these models cannot distinguish the role of muscle contractions and the role of gravitational accelerations for arterial adaptation. The HEPHAISTOS orthosis allows normal ambulation, while it significantly reduces force generation in the lower leg muscles. Eleven subjects wore HEPHAISTOS unilaterally for 56 days and were followed up for another 4 wk. Arterial diameters, intima media thickness (IMT), flow-mediated dilation (FMD), and resting blood flow (BF_{rest}) were measured using high-frequency ultrasonography. Arterial adaptations were investigated in the superficial femoral artery (SFA), the brachial artery (BA), and the carotid artery (CA). Mean SFA resting diameter was decreased from 6.57 mm (SD = 0.74 mm) at baseline to 5.77 mm (SD = 0.87 mm) at the end of the intervention ($P < 0.001$), whereas SFA wall-to-lumen ratio, SFA BF_{rest}, and SFA FMD remained unaffected throughout the study. The application of HEPHAISTOS had no effect on structure and function of the systemic control sites, the BA, and the CA. Our findings highlight the importance of muscular contractions for arterial diameter adaptations. Moreover, we propose that FMD and wall-to-lumen ratio are unaffected by ambulating with the HEPHAISTOS orthosis, which is suggestive of habitual acceleration profiles in the lower leg constituting an important stimulus for the maintenance of FMD and wall-to-lumen ratio.

gravitational impacts; arterial structure; arterial function

IT IS GENERALLY ACCEPTED that blood vessels, including larger arteries, adapt their structure as well as their functioning in response to alterations in their environment. In this context, it is mostly held, that arterial adaptations occur in response to mechanical stimuli such as shear rate, which is thought to be a primary load (10), acting on the endothelial layer (14, 29). Evidence suggests that endothelial cells (ECs) are able to sense shear rate as friction and dragging forces that are exerted on the cells of the vessel wall by blood motion (9). Alternative mechanical stimuli for arterial adaptation, which are being considered to be sensed by ECs and vascular smooth muscle cells (VSMC) are muscle shortening-related axial "stretch stresses," which are thought to stretch the adjacent tissues and blood vessels, and pressure-related circumferential wall stresses (10, 19).

Given the habitual activities in our gravitational environment, there must be four potential sources for mechanical stress acting on the arterial wall: *1*) muscle contractions provoking mechanical stretch and compression to the vasculature; *2*) phasic, blood flow-related pulsatile shear; *3*) blood pressure as the sum of hydrostatic and hydrodynamic pressure; *4*) gravitational accelerations induced by ground reaction force impacts.

To date, the specific role of gravitational accelerations on arterial adaptation has not been evaluated independently from mechanical stimuli induced by muscle work. Of note, walking and running are associated with vertical accelerations of up to 10 g (12), meaning that habitual everyday activities are likely to provide acceleration-related stresses on the arterial wall that are not directly depending on muscle contractions.

Chronic disuse such as bed rest, spinal cord injuries, spaceflight, and limb immobilization (ULLS) are associated with substantial adaptations of arterial structure and function (2, 3, 5, 26). These disuse models reveal that the general reduction of blood flow, as a consequence of muscular unloading, trigger the extensive arterial adaptations observed in immobilized subjects. However, none of these models is valid to independently investigate the effects of gravitational loading for arterial adaptation, since all established disuse models are characterized by both the extensive reduction of muscle work-related stresses and the absence of gravitational acceleration-related stresses. These studies also suggest that constant blood pressure changes cannot explain the long-term adjustment of arterial diameter and arterial function (2, 3).

Given the considerable number of diseased people who are temporarily or permanently immobilized, the study of the effect of such genuine gravitational forces on arterial structure and function could be very relevant for clinical applications in rehabilitation and prevention. Our current interest in this problem had been stirred by a new orthotic device that greatly reduces calf muscle activity and plantar flexion torque (Ducos M., Weber T., Albracht K., Messkemper J., Brüggemann GP, Rittweger J, unpublished observations) but maintains gravitational loading of the lower leg. Hence, we ventured to explore possible vascular adaptations that would emerge when wearing this new "HEPHAISTOS" orthosis for 8 wk. Using ultrasonography, we measured arterial diameters and intima media thickness (IMT) as structural parameters as well as resting blood flow (BF_{rest}) and flow-mediated dilation (FMD) as functional parameters. We hypothesized that, compared with the other well established disuse models, retention of habitual gravitational impacts in our new model would attenuate arterial diameter decrease, arterial wall thickening, and disuse-specific increase of FMD.

METHODS

Study Design, Intervention, and Subjects

The unloading orthosis. A novel unloading orthosis (Fig. 1; HEP HAISTOS, patent application no. 102011082700.5) has been devel-

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Fig. 1. The HEPHAISTOS unloading orthosis. A subject wearing HEPHAISTOS and the elevated plateau shoe on the contralateral leg.

oped in the German Aerospace Center (DLR) in Cologne, Germany (see Fig. 1). The HEPHAISTOS significantly reduces the activation and force production of the major calf muscles during locomotion activities while it completely retains body mass impacts during the stance phase of the gait. It is applied with an elevated plateau shoe (Fig. 1*B*) on the contralateral leg, without the support of crutches, and allows normal ambulation. Its biomechanical function and its unloading effects can be briefly explained by the fact that it reduces the plantar lever arm of the foot by ~35%. Consequently, it substantially reduces plantar flexor torque and muscle activation. Also, it allows normal ambulation by compensating achilles-tendon function by incorporating an elastic foot, which stores and releases kinetic energy during the stance phase of the gait. The biomechanical characteristics of the HEPHAISTOS orthosis have been comprehensively assessed by measuring muscle activation using EMG, by measuring reaction force impacts in and outside the orthosis using force plates and pressure insoles, by measuring plantarflexor torque using pressure insoles, and by investigating gait characteristics using the a motion-capture system. The following link leads to the webpage of the DLR showing a video with a subject walking with the HEPHAISTOS (http://www.dlr.de/me/en/desktopdefault.aspx/tabid-7389/).

The HEPHAISTOS study. The HEPHAISTOS study has been registered at clinicaltrials.gov (identifier: NCT01576081). It was designed as an integrative, one-group ambulatory interventional study where diverse physiological parameters were assessed. The intervention time was scheduled to 8 wk to enable a valid comparison with previous measurements (3). Eleven male subjects were recruited to wear the HEPHAISTOS unloading orthosis unilaterally. All subjects had been examined by a medical doctor before study inclusion. They also had to pass a psychological assessment including a standardized personality test [Freiburger personality inventory (FPI)] and a 45-min interview with two psychologists specialized in selecting flight personnel and study subjects. Exclusion criteria were any known disease or abnormality; any bone, tendon, or muscle injury during the last 12 mo; smoking; regular strength training; any regular medication. A written, informed consent was obtained from all subjects before commencement of the study. The HEPHAISTOS study was approved by the Ethics Committee of the Northern Rhine medical association (Ärztekammer Nordrhein, applilcation no. 2010169) in Duesseldorf.

A 1-€ coin (Bundesbank, Cologne, Germany) was tossed for each subject to determine which leg should be unloaded. The 11 subjects were familiarized with their individually adjusted orthosis 1 wk before the intervention started. The familiarization took ~1 h and was completed as soon as the subjects learned to walk naturally with the orthosis. For the 8 intervention weeks, subjects followed their normal everyday activities while wearing the device in all activities that required loading of the leg. Subjects had to visit the laboratory on a weekly basis for measurements and reports. After consulting the subjects, we estimated a "net wearing time" of 12–16 h/day, depending on their habitual activities. The anthropometric data of the subjects at baseline are presented in Table 1.

Procedures

Measurement protocol. Arterial diameter of BA and SFA, resting blood flow of BA and SFA, intima media thickness (IMT) of carotid artery (CA) and SFA, and flow-mediated dilation (FMD) of BA and SFA were examined by ultrasonography at baseline and at *days 5*, *28*, and *56* of the intervention, as well as after 5, 14, and 28 days of recovery (respectively, BDC, HEP5, HEP28, HEP56, R5, R14, and R28).

Measurements. Blood cell velocity and diameter measurements of the BA were performed using the Duplex mode of an echo Doppler device (Mylab25, esaote, Firenze, Italy), with a 12- to 18-MHz broadband linear transducer (LA 523). Blood cell velocity and diameter measurements of the SFA were performed in the Duplex mode using a 7.5- to 12-MHz broadband linear transducer (LA 435). For resting diameter measurements, videos with duration of ≥1 min were recorded for offline analysis. For FMD assessment of SFA and BA, a cuff was placed distal to the probe that was inflated to 300 mmHg for 5 min. Ten seconds before cuff deflation, video recording was started, and the FMD response was recorded for 5 min after cuff deflation. The IMT was determined by the IMT software tool (esaote, QIMT, for MyLab25). The IMT analysis tool processes the radio frequency (RF) signal from the ultrasound device in real time. IMT videos were recorded for ≥5 heart cycles using a 7.5- to 12-MHz broadband transducer placed parallel to the assessed artery. The region of interest (ROI) for IMT measurements was placed at the region of the artery with the highest image quality. Resting heart rate and blood pressure were measured before cuff inflation using an electronic sphygmomanometer (medicus pc, boso, Jungingen, Germany).

Subjects rested in a darkened room for at least 20 min in supine posture, fasted before the measurements for ≥8 h, and also refrained from caffeine, alcohol, and exercise for ≥8 h before the measurement. All measurements were performed by the same examiner. To avoid circadian variation, all measurements were performed at the same time of the day.

The angle of inclination for all Doppler velocity measurements was consistently adjusted to 60°, whereas the vessel area was set parallel to the transducer. The same placement of the probe for all conditions was assured by marking the skin above the artery of interest using anatomical landmarks, such as the upper patella edge for SFA and the radius epiphysis for the BA. All duplex videos were recorded on an external computer using the analog output of the device with a video grabbing system (GrabsterAV 450MX, Terratec, Nettetal, Germany) and analog-to-digital transformation software (MAGIX, Terratec, Nettetal, Germany).

Data Processing

Diameters and FMD. All videos were analyzed offline. Duplex video analysis was performed using custom-built edge-detection and wall-tracking software (Vasculometer 1.2; Ref. 4). The signal from the wall-tracking software was processed with MATLAB (Math-

Table 1. *Subject characteristics of the HEPHAISTOS study*

Number of subjects	11
Age, yr	31.1 ± 6.4
Body mass, kg	81.2 ± 10.0
Height, m	1.82 ± 0.06
Body mass index	24.6 ± 2.9
Systolic blood pressure, mmHg	119 ± 10
Diastolic blood pressure, mmHg	73 ± 8
Resting heart rate, beats/min	64 ± 5

works, Natick, MA), using a moving average filter with a span of 500 video frames. The median of all processed values before cuff deflation was taken as resting diameter. The highest value of the filtered signal was identified and used as peak diameter after cuff release. The FMD response was then expressed as the relative increase from resting diameter before cuff inflation to peak diameter after cuff deflation.

IMT. IMT video analysis was performed using a video sequence of ≥5 heart cycles. The IMT videos were analyzed offline, and the IMT value with the lowest standard deviation (≤20 μm), which was calculated by the QIMT software tool from esaote, was taken as IMT.

Blood flow. All blood flow measurements are based on the analysis of Duplex video sequences using the peak envelope of the Doppler waveform and the arterial diameters. The peak of the envelope of the Doppler waveform and the arterial diameters were automatically detected using the custom-built tracking software (4) and MATLAB software (Mathworks, Natick, MA) to process the tracking signal. The mean velocity (V_{mean}) and the corresponding diameter (D) were then used to calculate blood flow {$BF = [\pi(D/2)^2](V_{mean}/2)\cdot 60$}, with BF in ml/min, V_{mean} in cm/s, and D in cm. Resting blood flow was measured for 1 min in supine posture.

Statistical Analysis

Statistical analyses were performed using STATISTICA 8.0 for Windows (Statsoft, Tulsa, OK, 1984–2008). A repeated-measures ANOVA was performed with time (seven levels) as main factor for all FMD, IMT, IMT/lumen, resting diameter, heart rate, and blood pressure measurements. Tukey's test was used for post hoc testing. The results of the blood flow measurements were tested performing paired *t*-tests. Values are given as means ± SD. The significance level was set at $P \leq 0.05$.

RESULTS

Due to medical reasons, which were not related to the present intervention, one subject could not complete the study. The data of this subject are discarded from the analyses.

Diameter

SFA diameter decreased significantly ($P < 0.001$) from BDC to HEP56 by 12.7% (SD = 6.6%). Twenty-eight days after the intervention, SFA diameter reached baseline level again (Fig. 2*A*; $P = 0.92$ for BDC vs. R + 28). The intervention did not have an effect on resting diameter of the BA (Fig. 2*B*; $P = 0.92$).

IMT and Wall-to-Lumen Ratio

The thickness of intima and media of the SFA changed significantly over time during the HEPHAISTOS study (Fig. 2*C*; $P = 0.03$). However, post hoc testing did not reveal any significant difference between any particular time points. The ratio between SFA IMT and arterial lumen remained constant throughout the study (see Fig. 4; $P = 0.19$). The IMT of the CA was not affected by the intervention (Fig. 2*D*; $P = 0.8$).

FMD

No effect of time was observed for the FMD response of the SFA (Fig. 2*E*; $P = 0.32$) or for the FMD response of the BA (Fig. 2*F*; $P = 0.56$).

SFA Resting Blood Flow

Resting blood flow volume in the SFA remained unaffected after the HEPHAISTOS intervention (Fig. 3*C*; $P = 0.9$). The mean resting flow velocity in the SFA was significantly increased by 17% (SD = 21.5%) after the study (Fig. 3*B*; $P = 0.035$), whereas SFA diameter decreased significantly.

Resting Heart Rate and Blood Pressure

Resting heart rate ($P = 0.06$) as well as systolic ($P = 0.27$) and diastolic blood pressures ($P = 0.2$) remained unaffected during the intervention (Fig. 4).

DISCUSSION

The main aim of this study was to investigate the independent effects of accelerations during habitual physical activity on arterial structure and function. For this purpose, we developed a novel unloading orthosis that greatly reduces plantarflexor activation without reducing ground reaction forces during ambulation and tested it in a 56-day interventional study. The results of the HEPHAISTOS study suggest that gravitational accelerations alone are insufficient to maintain arterial structure. We observed a steady decrease of SFA resting diameter during the unloading phase, which is in line with the literature addressing the effects of the established disuse models (2, 3, 5). However, the present results for IMT, FMD, and BF at rest deviate from the above studies, revealing a potential impact of gravitational accelerations on vascular adaptation.

Changes of SFA Resting Diameter

Our main hypothesis was based on the view that *1*) mechanical stimuli (or the absence of mechanical stimuli) affect the resting diameter in conduit arteries and that *2*) habitual gravity-related accelerations would provide an effective stimulus to attenuate arterial resting diameter adaptations when muscle work-driven pulsatile shear rate is reduced.

Contrary to these assumptions, the results of this study suggest that the reduction of muscle work and the accompanied reduction of blood flow-related shear lead to a distinct decrease of arterial resting diameter, which is comparable to the diameter reduction in similar time frames under bedrest (8-wk HEPHAISTOS study, −12.7%, SD = 6.6% vs. bed rest, 17%, SD = 6.7%) (3) and limb suspension (4-wk HEPHAISTOS study, −10.9%, SD = 5.3 vs. ULLS, −12%, SD = 4%) (2) conditions, despite the fact that habitual gravitational accelerations remained unchanged in the present study. This finding is in line with the existing literature that supports the idea that blood flow-related endothelial shear acts as the main driver for conduit artery remodeling (1, 13, 29). However, the influence of gravitational accelerations for the resting diameter adjustment of conduit arteries cannot be entirely excluded. Recent findings in bedrest studies suggest that a combination of artificial high-frequency accelerations using a vibration plate and resistive exercise can attenuate the immobilization-induced resting diameter decrease, whereas resistive exercise alone was not sufficient to counteract this decrease (3, 31). On the other hand, the superposition of vibrations did not have a specific effect in healthy ambulatory subjects when combined with resistive exercise (33).

A possible way of reconciling the results of the above studies with the results of this study would be to conclude that gravitational accelerations have an impact on resting diameter adaptations of arteries if the following two conditions are fulfilled: *1*) gravitational accelerations have to be applied in combination with muscle contractions (3, 31) and *2*) the effect of gravita-

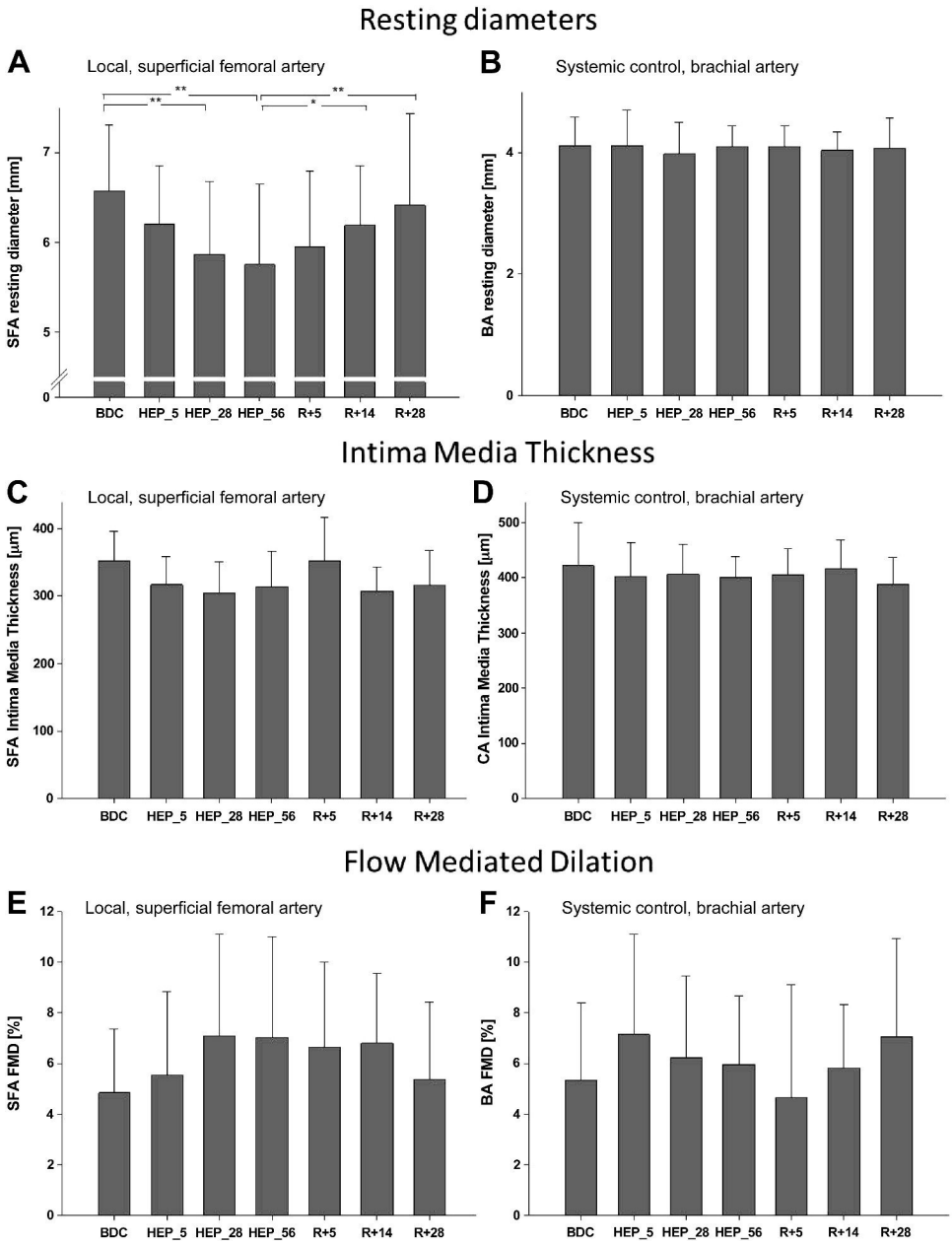

Fig. 2. Arterial parameters at rest. *A*: the time course of superficial femoral artery (SFA) resting diameter throughout the study. SFA diameter decreased continuously during the 8 intervention weeks. Resting diameter, as measured on the 56th day that the HEPHAISTOS orthosis was worn (HEP56), was 12.7% (SD = 6.6%) lower compared with baseline diameter (BDC; $P < 0.001$). After 4 wk of recovery, SFA resting diameter went back to BDC level [with $P = 0.92$ for BDC vs. 28 days of recovery (R + 28)]. *B*: BA resting diameter remained unaffected. *C*: the time course of SFA intima media thickness (IMT) changes. IMT of the SFA changed significantly over time ($P = 0.03$). *D–F*: carotid artery (CA) IMT (*D*) as well as SFA flow-mediated dilation (FMD; *E*) and bracheal artery (BA) FMD (*F*) remained unaffected during the study.

tional accelerations must not be saturated through habitual activities (33).

Changes of SFA IMT

The thickness of the intima and media of an artery is thought to provide an index of sub-intimal thickening and is commonly used as a surrogate marker for preclinical atherosclerosis (7). A thicker intima media layer is strongly associated with an increased risk for cardiac and peripheral vascular events, whereas a smaller IMT is associated with cardiovascular health (23).

However, the underlying mechanisms for arterial IMT adaptations are not entirely understood. Thijssen et al. (23) recently reviewed the considered exercise-specific stimuli for IMT adaptation. As for diameter remodeling too, mechanical hemodynamic stimuli such as shear rate and arterial pressure seem to play crucial roles for changes of IMT. An increase of blood flow-related shear rate is thereby associated with a reduction of IMT (24), whereas chronic increases in blood pressure are associated with arterial wall thickening (22). Apart from these, also systemic, nonhemodynamic stimuli, like vascular tone, sympathetic nervous system activity, oxidative stress, and inflammatory processes, seem to have an impact on arterial wall thickness (23).

As mentioned, the application of the HEPHAISTOS unloading orthosis is characterized by a significant local reduction of muscle force generation, hence by a local reduction of blood flow-related shear rate, while hydrostatic arterial pressure and gravitational loading remain unaltered. Consequently, the latter two characteristics deviate from two other investigated disuse models, spinal cord injury (SCI) and bed rest, which reported a systemic increase of IMT (16, 30).

The fact that IMT was reduced in the SFA but not in the CA largely excludes the possibility of a systemic effect during the

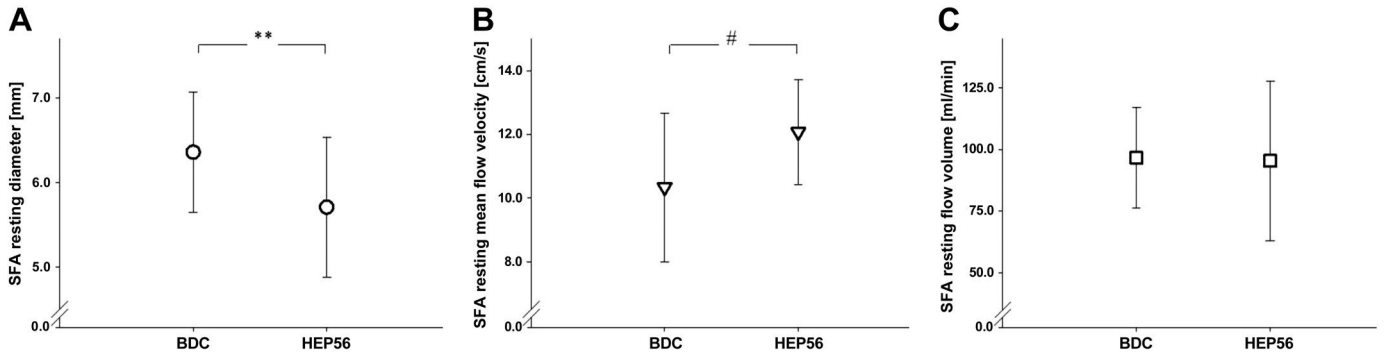

Fig. 3. Resting SFA blood flow parameters. SFA resting blood flow volume (*C*) was calculated using SFA resting diameter (*A*) and SFA resting mean velocity (*B*). The unchanged SFA resting flow volume at HEP56 ($P = 0.9$) results from the elevation of mean flow velocity (+17%, SD = 21.5%; $^{\#}P = 0.035$), whereas SFA resting diameter decreased significantly (−12.7%, SD = 6.6%; $^{**}P < 0.001$).

intervention. Conversely, the time course of IMT changes during our and other disuse studies suggest that the observed changes of IMT might be more attributable to changes in vascular tone than to actual atherosclerotic structural remodeling (27).

In light of this consideration, one could conclude that the present findings deviate from findings observed in bedrest and SCI. This could be for two reasons. *1*) The time course of SFA IMT changes represents changes of local vascular tone. Accordingly, gravitational accelerations, which are absent in bed rest and SCI, do provide a valid stimulus to reduce vascular tone. *2*) Habitual whole body activities maintain sympathetic nerve activity; hence, SFA and CA IMT did not (disuse specifically) systemically increase during the local HEPHAISTOS intervention.

Arterial Wall-to-Lumen Ratio

The finding of an unchanged arterial wall-to-lumen ratio of the SFA in this study is in stark contrast with bedrest, where wall-to-lumen ratio has been found to increase as a consequence of diameter decreases and IMT increases (30). It could well be that the provision of habitual whole body activity and the provision of habitual gravitational accelerations lead to adjustments of vascular tone, which in turn lead to an equilibrium between arterial wall and arterial lumen (see discussion above).

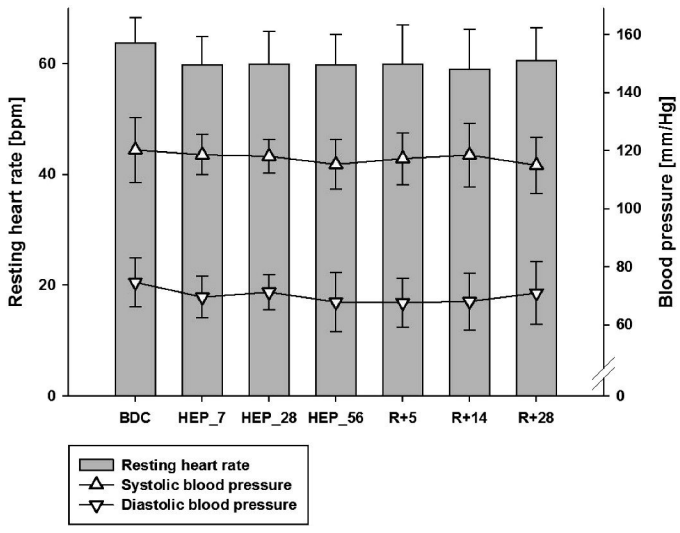

Fig. 4. Resting heart rate and blood pressure. Resting heart rate was measured in supine posture before the first cuff inflation. There were no significant changes for all parameters: heart rate ($P = 0.06$), systolic ($P = 0.27$), and diastolic blood pressure ($P = 0.2$).

FMD

Typically, muscular disuse is associated with an increase of FMD, which is being used as a measure for endothelial function (2, 3, 5, 30). Both the larger shear stress stimulus occurring in smaller arteries and an increased sensitivity of smooth muscles to NO are being considered as reasons for an increased FMD after physical inactivity (25). Nonetheless, we found in our study that SFA FMD remained unaffected, whereas SFA diameter showed a distinct inward remodeling during the unloading phase.

Thijssen et al. (28) recently discovered an interesting interaction between arterial structure and arterial function. They found that arterial wall-to-lumen ratio and FMD response

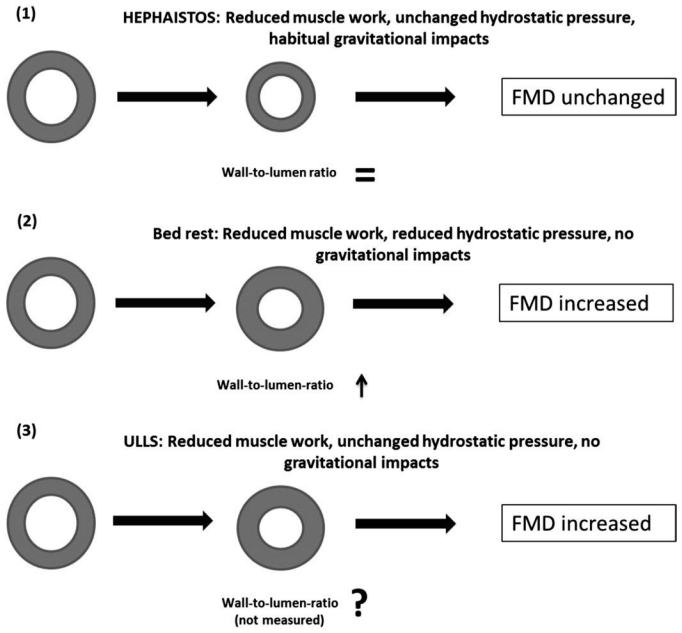

Fig. 5. Schematic overview. Simplified illustration of structural and functional SFA adaptations as a consequence of *1*) the HEPHAISTOS intervention, *2*) bed rest, and *3*) ULLS unloading.

significantly correlated in the investigated arteries of different size across the body. The idea is that a thicker media layer, which consists of smooth muscle cells, would provide an increased dilation potential. Accordingly, one possible explanation for the unchanged FMD of the SFA in the present study could be that SFA wall-to-lumen ratio remained constant during the study. Consequently, a possible conclusion would be that the ratio of arterial wall and lumen is more important for FMD adjustment than the magnitude of shear rate.

SFA Blood Flow

Notwithstanding the distinct inward remodeling during the present study, SFA resting blood flow volume could be maintained after the HEPHAISTOS intervention. The retention of SFA flow volume is achieved by the elevation of mean flow velocity. The present findings are in agreement with previous studies of deconditioning, where arterial resting blood flow was found to be unchanged after a period of unilateral limb suspension (2), after SCI (6), and after bed rest (3).

In conclusion, the above findings as well as the findings of exercise studies (8, 20, 33) support the contention of Laughlin et al. (14) that the most important muscle work-related signal for endothelial cells is constituted by the increased shear stress due to the increase of regional blood flow to provide working muscles with oxygen. Accordingly, the elevated resting shear rate due to diameter decreases and concomitant flow velocity increases, as observed after muscle unloading, does not account for the adjustment of resting conduit artery diameter.

Resting Heart Rate and Blood Pressure

Changes of resting heart rate strongly correlate with the magnitude of physical activation. As seen in hypokinesia and exercise training, resting heart rate has been reported to progressively increase due to physical inactivity (11, 17) and to decrease in response to increases of physical activity (21). Physical activity is also thought to decrease arterial blood pressure (32), whereas previous hypokinetic studies reveal diverse blood pressure adaptations (15, 18). However, the finding that resting heart rate as well as systolic and diastolic blood pressure did not change for any time point of the present study suggests that subjects maintained their habitual physical activity during the HEPHAISTOS intervention and during recovery.

In conclusion, 8 wk of muscular lower leg unloading with unchanged habitual acceleration profile led to significant site-specific adaptations in SFA diameter. However, we did not observe a disuse specific increase of wall-to-lumen ratio. Furthermore, the FMD response of the investigated arteries seemed to remain unaffected during the intervention. These findings are at variance with findings in bedrest, ULLS, and SCI, where FMD and diameter were always inversely affected (see Fig. 5). Based on these data, we propose that FMD is unaffected by ambulating with the HEPHAISTOS orthosis, which is suggestive of habitual acceleration profiles in the lower leg constituting an important stimulus for the maintenance of FMD.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: T.W., M.D., E.M., G.-P.B., W.B., and J.R. conception and design of research; T.W., M.D., E.M., and F.H. performed experiments; T.W. and F.H. analyzed data; T.W. and J.R. interpreted results of experiments; T.W. prepared figures; T.W. drafted manuscript; T.W., M.D., E.M., W.B., and J.R. edited and revised manuscript; T.W., W.B., and J.R. approved final version of manuscript.

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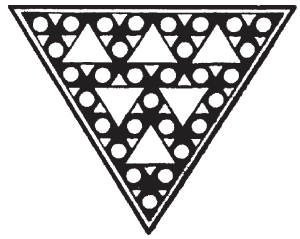

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The relationship between exercise-induced muscle fatigue, arterial blood flow and muscle perfusion after 56 days local muscle unloading

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arterial blood flow; muscle fatigue; muscle perfusion; muscle power; muscle unloading

Summary

In the light of the dynamic nature of habitual plantar flexor activity, we utilized an incremental isokinetic exercise test (IIET) to assess the work-related power deficit (WoRPD) as a measure for exercise-induced muscle fatigue before and after prolonged calf muscle unloading and in relation to arterial blood flow and muscle perfusion. Eleven male subjects (31 ± 6 years) wore the HEPHAISTOS unloading orthosis unilaterally for 56 days. It allows habitual ambulation while greatly reducing plantar flexor activity and torque production. Endpoint measurements encompassed arterial blood flow, measured in the femoral artery using Doppler ultrasound, oxygenation of the soleus muscle assessed by near-infrared spectroscopy, lactate concentrations determined in capillary blood and muscle activity using soleus muscle surface electromyography. Furthermore, soleus muscle biopsies were taken to investigate morphological muscle changes. After the intervention, maximal isokinetic torque was reduced by 23·4 ± 8·2% ($P<0·001$) and soleus fibre size was reduced by 8·5 ± 13% ($P = 0·016$). However, WoRPD remained unaffected as indicated by an unchanged loss of relative plantar flexor power between pre- and postexperiments ($P = 0·88$). Blood flow, tissue oxygenation, lactate concentrations and EMG median frequency kinematics during the exercise test were comparable before and after the intervention, whereas the increase of RMS in response to IIET was less following the intervention ($P = 0·03$). In conclusion, following submaximal isokinetic muscle work exercise-induced muscle fatigue is unaffected after prolonged local muscle unloading. The observation that arterial blood flow was maintained may underlie the unchanged fatigability.

Introduction

Disuse-induced adaptations of skeletal muscle are manifold. Not only is there muscle atrophy and a fibre-type shift towards more glycolytic type II fibres with a lower endurance capacity (Trappe *et al.*, 2004; Degens & Alway, 2006), but there are also changes in electromyographic activity (Mulder *et al.*, 2007) as well as distinct structural and functional adaptations of blood vessels supplying the unloaded muscles (Thijssen *et al.*, 2010).

As blood vessels are able to rapidly adjust to altered functional demands and considering that peripheral blood flow is dependent on the vasculature, adaptations of structure and function of blood vessels that reduce blood flow must be considered to limit the ability to perform on-going muscle contractions and thus to increase exercise-induced muscle fatigability. Muscle fatigue is a general phenomenon that has been previously assessed in different ways (Enoka & Duchateau, 2008) and partly explainable as a result of the above adaptations, muscle performance in terms of maximal force output and exercise-induced muscle fatigue has indeed been found to be impaired after prolonged disuse (Mulder *et al.*, 2007). The disuse-induced increase of muscle fatigue is, however, not unequivocal, as various studies have found no

effect (Witzmann *et al.*, 1983; Koryak, 1996) or even a decreased fatigability (Semmler *et al.*, 2000; Shaffer *et al.*, 2000) after muscle unloading. Some parts of the discrepancies between studies may be related to different models of disuse, investigated parameters and exercise protocols. In addition, exercise-induced muscle fatigue as studied in previous research (Koryak, 1996; Portero *et al.*, 1996; Semmler *et al.*, 2000; Mulder *et al.*, 2007) was predominantly investigated performing sustained isometric contractions where blood flow is already occluded at comparably low torque levels (de Ruiter *et al.*, 2007) or performing intermittent isometric contractions (Witzmann *et al.*, 1983; Koryak, 1996; Mulder *et al.*, 2007). These studies did not consider the dynamic nature of the majority of daily locomotive muscle contractions. Other human studies have investigated exercise-induced muscle fatigue under dynamic conditions after disuse did not investigate parameters for arterial blood supply and muscle perfusion (Berg *et al.*, 1993; Deschenes *et al.*, 2002) and final conclusions about the specific impact of blood supply on changes of exercise-induced muscle fatigue under dynamic conditions after periods of muscle disuse cannot be made.

Consequently, for the purpose of the present work, it should be investigated in how dynamic contractions and moderate work rate would affect muscular power generation after prolonged local muscle unloading. Local exercise-induced fatigue was thus assessed calculating the work-related power deficit (WoRPD) during a standardized local exercise test. This test was specifically developed to reflect habitual calf muscle contractions where a steady blood supply allows for enduring muscle work.

Yet, if reductions in blood supply to locomotive muscles during muscle disuse contribute to dynamic exercise intolerance, this holds great clinical potential to develop effective preventive measures in disease and injury rehabilitation in conditions associated with muscle unloading, aiming at maintaining local circulation (e.g. low-intensity exercise or thermotherapy). Therefore, the aim of the present study was to investigate the relationship between blood supply and isokinetic WoRPD after a period of local muscle unloading. Local disuse adaptations in calf muscle blood supply and WoRPD were studied using the HEPHAISTOS unloading orthosis that greatly reduces calf muscle force production during the stance phase without altering the gait pattern (Weber *et al.*, 2013; Ducos M, Weber T, Albracht K, Brüggemann G-P, Rittweger J, manuscript in revision). Previous whole body (Huonker *et al.*, 2003; Bleeker *et al.*, 2005b; De Groot *et al.*, 2006) and local disuse studies (Shaffer *et al.*, 2000; Sugawara *et al.*, 2004; Bleeker *et al.*, 2005a) have found that the vasculature adapts distinctly, structurally as well as functionally to unloading. However, these studies did not elaborate on the consequences of the disuse-induced vascular adaptations with regard to exercise-induced muscle fatigue in terms of a WoRPD.

It was in the light of the above considerations the aim of the present study to comprehensively investigate changes of local blood supply and its potential impact on exercise-induced muscle fatigue after prolonged muscle unloading. In order to investigate the functional muscle capacity during an incremental isokinetic exercise test (IIET) that was performed before and after the unloading intervention, isokinetic plantar flexor torque was continuously recorded and muscle power was calculated. Further, neuronal changes after muscle unloading should be detected measuring electromyographic soleus muscle activity during the exercise test, while femoral artery blood flow (ultrasonography), blood lactate concentrations and soleus muscle tissue oxygenation (near-infrared spectroscopy) were measured to assess changes of blood supply and metabolic properties of the unloaded muscle. In addition, before and after the HEPHAISTOS intervention, muscle biopsies were taken from the soleus muscle and histochemically analysed to assess fibre-type distribution and muscle capillarization.

Thus, the primary hypothesis of the present study was that after 8 weeks of local muscle unloading, the local blood flow at a given relative submaximal workload is reduced. We further expected a priori that if blood flow would be reduced, the reduction of blood supply would lead to an increase of WoRPD under isokinetic conditions.

Methods

Participants

Before study inclusion, subjects underwent comprehensive medical and psychological examinations. Prior to commencement of the study, a written informed consent was obtained from all subjects. The HEPHAISTOS study was approved by the Ethics Committee of the Northern Rhine medical association (Ärztekammer Nordrhein, Duesseldorf, Germany).

Procedures

Unloading orthosis

In order to inactivate the calf muscles during locomotion, subjects wore the HEPHAISTOS orthosis in all daily activities that required loading of the legs (Fig. 1, patent application number 102011082700.5). The orthosis allows normal ambulation while activation and force production of the major calf muscles are significantly reduced, whereas the impact of ground reaction forces is completely retained. The biomechanical principles and acute effects of wearing the HEPHAITOS are published elsewhere (Ducos M, Weber T, Albracht K, Brüggemann G-P, Rittweger J, manuscript in revision). In short, HEPHAISTOS reduces the plantar lever arm of the foot by approximately 35%, while ground reaction forces are retained. This leads to a substantial reduction of plantar flexor activation and plantar flexor torque production, in particular of the soleus muscle. A natural gait pattern can be maintained through the function of the elastic foot underneath the sole, which stores and releases energy during gait much like the

Figure 1 HEPHAISTOS. A subject wearing the HEPHAISTOS unloading orthosis and the elevated contralateral plateau shoe.

Achilles tendon. The link below leads to the DLR Space Physiology webpage where a video of a subject walking with HEPHAISTOS is presented (http://www.dlr.de/me/en/desktopdefault.aspx/tabid-7389/12432_read-35410/).

HEPHAISTOS intervention

A detailed description of the study design of the HEPHAISTOS intervention will be published elsewhere (Weber *et al.*, manuscript in revision). The study has been registered at www.clinicaltrials.gov (NCT01576081). Briefly, the HEPHAISTOS study (HEP-study) was conducted as an integrative single-group ambulatory interventional study. Eleven healthy male subjects (31 ± 6 years) wore the HEPHAISTOS unloading orthosis unilaterally for 56 days, while on the other leg, a shoe with an elevated sole of the same height was worn. During the study, participants visited the laboratory for measurements and reports on a weekly basis.

Isokinetic incremental exercise test

An exercise test was performed at baseline data collection (BDC) and on the last day of the intervention (HEP56) that was thought to be challenging, but not impossible to complete after 56 days HEPHAISTOS unloading. An incremental exercise design was chosen to ensure valid ultrasound measurements during the moderate stages in order to test the primary hypothesis and to enforce a work-related power deficit following the higher increments in order to test the secondary hypothesis. To allow investigations of WoRPD characteristics independently of changes related to maximal strength losses, submaximal target torque stages were normalized to the current maximal voluntary contraction (MVC) strength. While lying in supine position with the foot attached to a dynamometer (Biodex system 3; Biodex Medical Systems, Shirley, NY, USA), subjects performed four incremental exercise stages that were, based on pilot study results, set to 30%, 40%, 45% and 50% of the current isokinetic maximum voluntary contraction strength (MVC_R), which in turn was assessed prior to the exercise test. Each stage consisted of 40 submaximal contractions, followed by two maximal isokinetic plantar flexor contractions. Foot dorsiflexion was performed passively with external support. Between successive stages, subjects rested for an interval of 5 s. Angular velocity was set to 20 deg s^{-1} and the total movement angle ranged from -5 deg dorsiflexion to 15 deg plantar flexion, where 0 deg refers to the neutral position. To assess the reference MVC (MVC_R), subjects performed two sets of five maximal contractions per set, with 1-min pause between sets. The incremental submaximal stages were then set as a fraction of the MVC_R. During the IIET, subjects performed two MVCs before the first stage and two MVCs at the end of each stage. The highest power of the two MVCs at the end of a stage was used to assess WoRPD, given as a percentage power difference from MVC_R. For all MVC assessments, subjects were asked to produce as much plantar flexor torque as possible during verbal encouragement. Real-time visual feedback of the produced torque was provided to ascertain correct contraction strength for each submaximal stage. A schematic overview of the exercise protocol, including all measurements, is depicted in Fig. 2.

Functional measurements

Isokinetic measurements

Plantar flexor torque (τ) was recorded during the entire exercise protocol using the internal software of the Biodex3 dynamometer and a sampling frequency of 100 Hz. Peak torques were then determined offline for each MVC. Angular velocity (ω) was set to 20 deg s^{-1} (0·3491 rad s^{-1}) for all torque measurements, and mechanical power (P) was then calculated as: $P = \tau \cdot \omega$, with τ in Nm and ω in rad s^{-1} and Pin Nm s^{-1}.

Arterial blood flow

Blood flow (BF) was measured in the superficial femoral artery (SFA) using a Doppler ultrasound device (Mylab 25; Esaote, Firenze, Italy) with a 7·5 to 12 MHz broadband linear

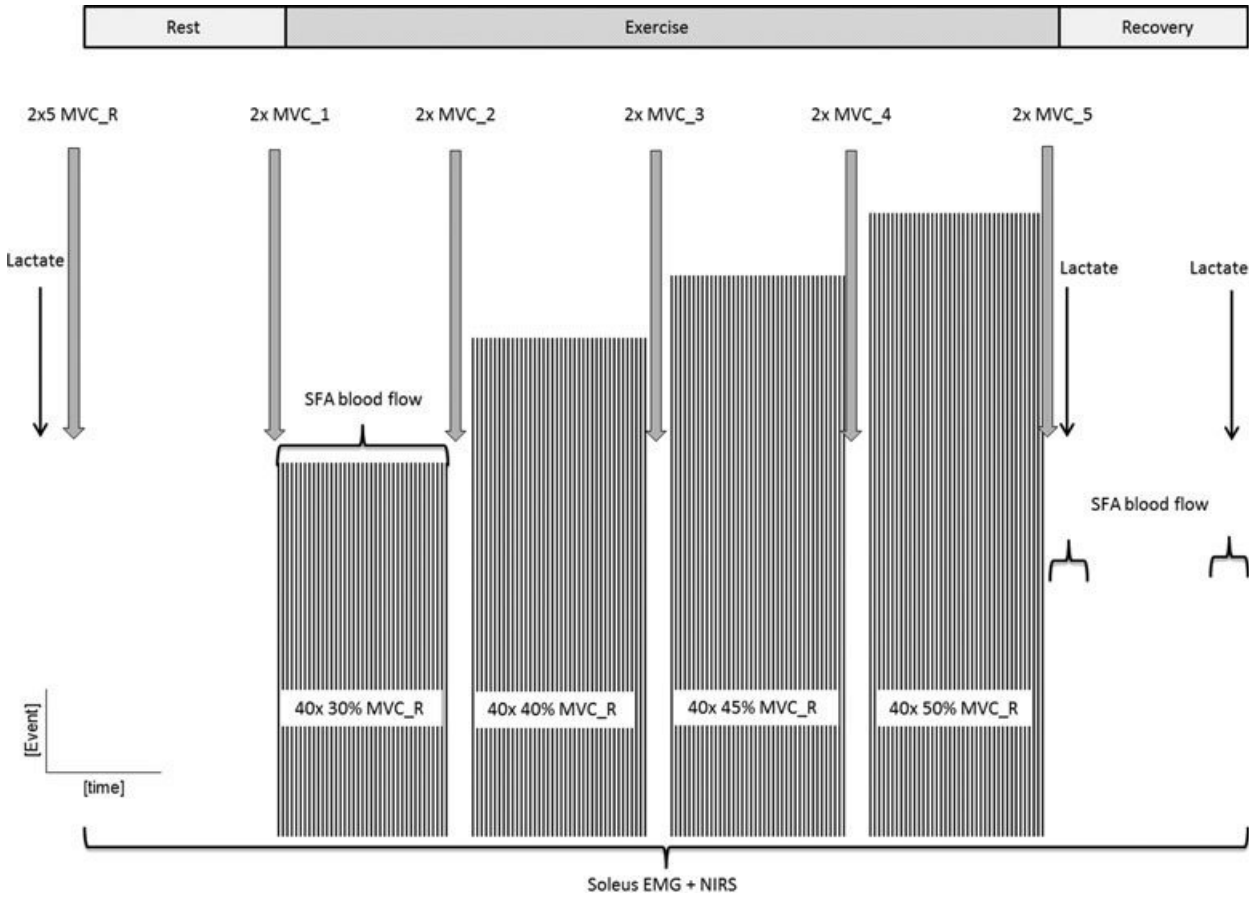

Figure 2 Exercise protocol. Schematic overview of the isokinetic incremental exercise test (IIET) including all measurements that were performed. MVC, maximal voluntary contraction; SFA, superficial femoral artery; EMG, electromyography; NIRS, near-infrared spectroscopy.

transducer. Resting blood flow (BF_{rest}) was measured in the morning under standardized conditions: subjects were asked to fast, refrain from alcohol, caffeine and exercise for ≥8 h prior to the measurement. Throughout the IIET, blood flow was measured during the 30% exercise stage ($BF_{exercise}$), directly after the last stage of the protocol (BF_{rec1}) and after 2 min of recovery (BF_{rec2}). The Duplex mode was used to simultaneously measure arterial diameter and blood flow velocity. The angle of inclination for Doppler measurements was set to 60 deg where the probe was placed parallel to the longitudinal section of the artery. Ultrasound videos were recorded on an external computer using the analogue output of the device and a video-grabbing system (GrabsterAV 450MX; Terratec, Nettetal, Germany) together with an analogue to digital transformation software (MAGIX; Terratec, Nettetal, Germany). Offline analysis of the recorded videos was performed applying custom-built software (Bremser *et al.*, 2012). Arterial blood flow was calculated using the envelope of the Doppler signal and the corresponding SFA diameters. Mean flow velocity (V_{mean}) and the corresponding artery diameter (D) were then used to calculate blood flow for each condition as: $BF = \pi\ (D{\cdot}0{\cdot}5)^2{\cdot}(V_{mean}{\cdot}0{\cdot}5){\cdot}60$, with BF in ml ml^{-1}, V_{mean} in cm s^{-1} and D in cm. Exercise-induced dilation was calculated as the relative diameter increase from rest.

Blood supply/mechanical power ratio

The blood flow values (ml min^{-1}) for the 30% MVC stage ($BF_{exercise}$) and the corresponding submaximal plantar flexor power (Nm s^{-1}) were taken to calculate the ratio of blood supply and mechanical power (BF/P), with BF/P in ml Nm^{-1}.

Muscle tissue oxygenation

Near-infrared spectroscopy was used during the entire experiment using a custom-made device (RheinAhrCampus Remagen of the Koblenz University of Applied Sciences). This device consists of a slow scan camera (model 7358-0003; Princeton Instruments, Roper Scientific, Trenton, NJ, USA), a detector chip with 1340 × 400 pixels and 16 bit resolution, a controller unit (model Spec-10; Princeton Instruments, Roper Scientific) and a spectrometer (model SP-150; Acton optics and coatings; Princeton Instruments, Trenton, NJ, USA). Details about the mode of operation of this device have been published elsewhere (Geraskin *et al.*, 2009). Tissue oxygenation index (TOI) was measured at the distal medial side of the soleus muscle using a sampling rate of 1 Hz. The median soleus muscle TOI was determined from data acquired 1 min before the IIET (TOI_{rest}) and for 2 min after the IIET

($TOI_{recovery}$). The minimal TOI was determined using the full period of the incremental test ($TOI_{exercise}$).

Electromyography

Soleus muscle surface EMG was obtained using a telemetric device (Trigno Wireless; Delsys Inc., Boston, MA, USA) applying the Seniam recommendations for surface electromyography (www.seniam.org). Electromyographic recordings were obtained throughout the entire IIET protocol using a sampling frequency of 4000 Hz. The signal was offline rectified and high pass-filtered (>50 Hz) with MATLAB (Mathworks, Natick, MA, USA). Submaximal contractions were detected by applying a threshold equivalent to 30 times the standard deviation of the EMG signal at rest. After visual inspection of the signal, incorrectly detected contractions were not considered. Subsequently, root mean square (RMS) and median frequency (MF) were calculated for each submaximal contraction. Values for RMS and MF of missing contractions were interpolated using the Piecewise Cubic Hermite Interpolating Polynomial (pChip function, MATLAB library). Means of RMS and MF were then calculated for all IIET stages (Fig. 7).

Lactate measurements

Blood lactate concentration was assessed in capillary blood taken from the ear lobe before, directly after and 2 min after the IIET protocol (LA_{rest}, LA_{rec1}, LA_{rec2}, respectively). The lactate concentration was analysed using a portable lactate analyser (Lactate Pro; Arkray, Kyoto City, Japan).

Histochemical analysis

Biopsy sampling

Biopsy samples from soleus muscle were collected after overnight fasting, both at baseline and on the 50th day of the immobilization phase in order to assure uncompromised functional data acquisition at HEP56. Biopsies were taken from the lateral side of the muscle, approximately 1 cm below the belly of the lateral gastrocnemius muscle. After skin disinfection and local anaesthesia (2–3 ml of 2% Lidocaine), skin and muscle fasciae were incised for 10 mm and muscle samples were taken with a Weil–Blakely rongeur (Gebrüder Zepf Medizintechnik, Tuttlingen, Germany). Samples were, under rapid shaking, immediately frozen in liquid nitrogen and subsequently stored at −80°C for further analyses.

Lectin staining of capillaries

Ten-μm thick cross-sections of soleus muscle biopsies were cut in a cryostat. Capillaries were stained with lectin (*Ulex Europaeus*): sections were fixed in ice-cold acetone for 15 min and washed in HEPES buffer. Natural occurring peroxidase activity was blocked, and after washing in HEPES, sections were incubated in lectin solution (50 μg ml^{-1} in HEPES). The location of the capillaries was revealed with 40 min ABC-staining solution (ABC, Vectastain; Vector Laboratories, Burlingame, CA, USA) followed after wash steps, by incubation with DAB (DAB substrate kit; Vector Laboratories) and embedded in glycerine gelatine.

Myosin ATPase staining

Serial sections were stained for myosin ATPase according to Brooke & Kaiser (1970). Briefly, sections were preincubated in sodium acetate solution (pH: 4.35), washed, incubated in alkaline buffer (pH: 9.4), washed, incubated in cobalt chloride solution (2%), washed, incubated in ammonium sulphide solution (1%), washed and mounted in glycerine gelatine. Type I fibres appear dark and type II fibres light (Fig. 3).

Analysis of stained sections

Whole sections were photographed with a 20-fold magnification using a light microscope (Axio Scope.A1; Carl Zeiss Microscopy GmbH, Göttingen, Germany) and a USB-Monochrome camera with a 1280 × 960 pixel chip (ICX205AL; Sony Corporation, Tokyo, Japan). Lectin-stained images were then analysed using the custom-made 'HISTOMETER' software (Fig. 3), which is implemented as plugin into the IMAGEJ image processing software (ImageJ 1.46r; National Institute of Health, Bethesda, MD, USA). Regions of interest (ROIs) were determined in the area of the muscle section with predominantly polygonal or circular-shaped muscle fibres. Fibres that were sectioned longitudinally were avoided in the analysis. Based on pixel analyses within a given ROI smallest fibre diameters (DiaMin), fibre cross-sectional areas (FCSA), capillaries around fibres (CaF), capillary density (CD) and capillary-to-fibre ratio (C/F) were determined. DiaMin was calculated as the smallest diameter (in μm) of each fibre polygon that crosses the polygon centre of gravity (Fig. 3), FCSA was calculated as the sum of all pixels within one polygon (in μm^2), CaF was calculated as the number of capillaries that were in direct contact with the fibre polygon (distance from capillary to fibre <9·3 μm), CD as the overall number of capillaries divided by the area of the entire ROI and C/F was calculated as the overall number of capillaries divided by the overall number of fibres. Finally, fibre-type distribution was assessed as the relative distribution of type I and type II fibres, and fibre area distribution as the relative area occupied by either fibre type. The average number of analysed fibres per ROI and section was 135 (SD = 48). All image analyses were performed by the same investigator.

Statistical analysis

Statistical analyses were performed using STATISTICA 8.0 for Windows (Statsoft, Tulsa, Oklahoma, USA, 1984–2008). A repeated-measures ANOVA was performed with four time levels (rest, exercise, rec1 and rec2) and two groups (BDC,

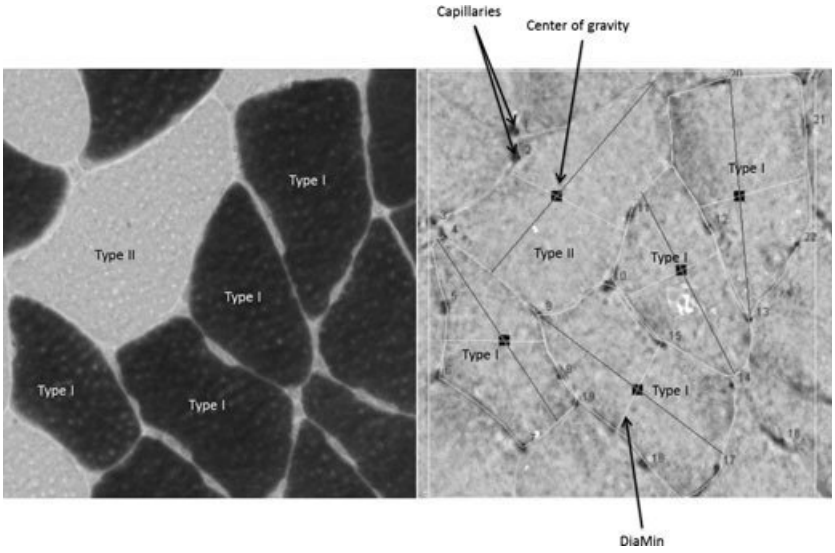

Figure 3 Soleus muscle sections. Left: myosin ATPase staining. Right: lectin staining for capillaries. Fibre types were transferred from myosin ATPase sections. Fibre polygons and capillaries were then analysed using custom-built software. Numbers are assigned for each object by the software. Note that for each muscle fibre DiaMin crosses the polygon centre of gravity.

HEP56) to detect changes in arterial blood flow and exercising blood flow velocity. Exercise-induced dilation was tested in the same way with three different time levels (exercise, rec1 and rec2). Soleus muscle oxygenation (TOI_{rest}, $TOI_{exercise}$, $TOI_{recovery}$) and lactate concentrations (LA_{rest}, LA_{rec1}, LA_{rec2}) were analysed with three different time levels and two groups (BDC, HEP56). In order to assess changes of MVCs within the IIET protocol, a repeated-measures ANOVA was performed with six time levels (MVCR–MVC5) and two groups (BDC and HEP56). Electromyography data were analysed with four time levels ($EMG_{30\%MVC}$, $EMG_{40\%MVC}$, $EMG_{45\%MVC}$ and $EMG_{50\%MVC}$) and two groups (BDC and HEP56). Tukey's test was used for post hoc testing. Pre-post-differences BF/P ratios as well as soleus muscle biopsy data were analysed with paired t-tests. Values are expressed as means ± SD. The significance level was set at $P \leq 0{\cdot}05$.

Results

Due to reasons unrelated to the HEPHAISTOS intervention, one subject could not attend the HEP56 IIET. Nonetheless, soleus muscle biopsies of this subject were taken as scheduled and the data were taken into account for soleus muscle morphology analysis. Electromyography data of two subjects had to be discarded from analysis due to technical failure. Superficial femoral artery blood flow could only be measured at rest, during the moderate 30% MVC stage of the IIET and after the IIET and not, as it was initially planned and tested before on experienced investigators, during the entire IIET. Whole body motion artefacts generally precluded sufficient Duplex ultrasound measurements with the relatively inexperienced subjects during higher torque levels.

Calf muscle performance

Absolute reference plantar flexor MVC torque (MVC_R) was significantly ($P<0{\cdot}001$) reduced by 23·4% (SD = 8·2%) at HEP56 compared with the BDC value (Fig. 4a). During the IIET, MVC power declined significantly (time: $P<0{\cdot}001$) from 49·9 Nm s^{-1} (SD = 6·8 Nm s^{-1}) to 36·8 Nm s^{-1} (SD = 6·7 Nm s^{-1}) at BDC and from 38·3 Nm s^{-1} (SD = 6·9Nm s^{-1}) to 27·2 Nm s^{-1} (SD = 4·8 Nm s^{-1}) at HEP56 (Fig. 4b). The IIET-related power reductions on both days were comparable when expressed as per cent decline (group: $P = 0{\cdot}88$; Fig. 4c).

Arterial blood flow parameters

Blood flow increased significantly in response to the IIET (time: $P<0{\cdot}001$) from 96 ml min^{-1} (SD = 27 ml min^{-1}) at rest (BF_{rest}) to 250 ml min^{-1} (SD = ml min^{-1}) for $BF_{exercise}$ and to 364 ml min^{-1} (SD = ml min^{-1}) until BF_{rec2}, that is, 2 min after termination of the IIET. Absolute SFA blood flow did not change after the intervention for any of the four tested time levels (Fig. 5a), as indicated by the absence of a significant group effect ($P = 0{\cdot}95$). Mean SFA blood flow velocity increased significantly in response to the IIET (Fig. 5b; time: $P<0{\cdot}001$) with no significant differences between BDC and HEP56 (group: $P = 0{\cdot}16$). Resting and exercising SFA diameters were significantly smaller at HEP56 (Fig. 5b; group: $P = 0{\cdot}03$) compared with BDC. In response to the IIET, SFA diameter dilated significantly (time: $P = 0{\cdot}002$) by 5·8% (SD = 7·5%) from rest to 2 min recovery (rec2) for the pooled data of BDC and HEP56. There is trend (group: $P = 0{\cdot}07$) that HEP56 exercise dilation was more pronounced than BDC exercise dilation.

Blood supply/mechanical power ratio

The ratio between SFA blood flow during the 30% MVC stage and the corresponding plantar flexor power (BF/P) increased significantly ($P = 0{\cdot}0046$) from 0·27 ml Nm^{-1} (SD = 0·06 ml Nm^{-1}) at BDC to 0·39 ml Nm^{-1} (SD = 0·11 ml Nm^{-1}) at HEP56 (Fig. 5d).

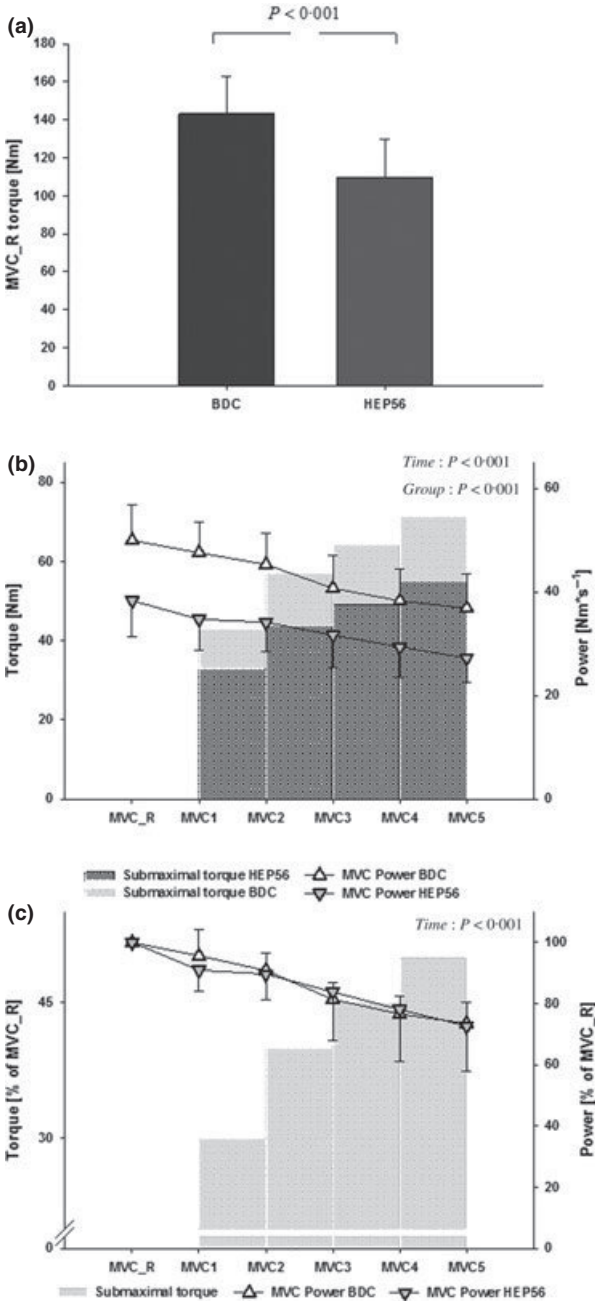

Figure 4 Isokinetic plantar flexor performance. Panel (a) depicts the significant (P<0·001) decrease of 23·4% (SD = 8·2%) of absolute peak isokinetic plantar flexor torque (MVC_R) after the intervention. The work-related power deficit is depicted in absolute values (b) and as percentage decrease from MVC_R (c). Vertical bars depict submaximal work stages with the corresponding target torques; with MVC_R as reference MVC and MVC1 to MVC5 as MVCs within the IIET. There was no difference of the WoRPD between BDC and HEP56 (P = 0·88).

Soleus muscle tissue oxygenation

Soleus muscle TOI was not significantly different during the IIET at HEP56 compared with BDC (group: P = 0·65). In response to the IIET, soleus muscle TOI decreased significantly (time: P<0·001) from 55·8% (SD = 2·9%) at rest (TOI_{rest}) to 50·8% (SD = 5·2%) during the IIET ($TOI_{exercise}$) and returned to baseline (56·0%; SD = 2·8%) during the 2 min recovery phase ($TOI_{recovery}$; Fig. 6).

Electromyography

In response to the IIET, soleus muscle EMG MF decreased significantly (time: P = 0·04) from 111 Hz (SD = 28 Hz) during the 30% MVC stage to 101 Hz (SD = 15 Hz) during the 50% MVC stage. There is a trend (group: P = 0·06) that overall MFs were reduced after the intervention; however, the decrease in response to the IIET was comparable between BDC and HEP56 (group*time: P = 0·81). The amplitude of the EMG signal increased significantly in response to the IIET as indicated by an increased RMS throughout the experiment (time: P<0·001). The increase of RMS by 109% (SD = 68%) from the 30% MVC stage to the 50% MVC stage in response to the BDC IIET appeared to be significantly more pronounced compared with the 67% (SD = 57%) increase in response to the HEP56 IIET (group*time = 0·03; Fig. 7).

Lactate concentration

There was no difference between BDC and HEP56 capillary blood lactate concentration (group: P = 0·13). In response to the IIET, lactate concentration increased significantly (time: P<0·001) from 1·3 mmol l^{-1} (SD = mmol l^{-1}) at rest to 2·2 mmol l^{-1} (SD = 0·59 mmol l^{-1}) directly after the IIET and to 2·3 mmol l^{-1} (SD = 0·62 mmol l^{-1}) 2 min after the IIET.

Soleus muscle morphology

Across fibre types, fibre size (DiaMin) was significantly (P = 0·016) reduced by 8·5% (SD = 13%) after the intervention. Fibre-type specific analysis of DiaMin revealed only a significant reduction (P = 0·031) in type I fibre diameter (−11%, SD = 14%). The FCSAs of type I fibres trended (P = 0·06) to be reduced following the intervention. Across fibre types, the mean number of CaF decreased significantly (P = 0·023) from 4·2 (SD = 1·3) to 3·6 (SD = 0·6). Capillary density (P = 0·16), capillary-to-fibre ratio (P = 0·53), fibre-type distribution (P = 0·96) and FCSA distribution (P = 0·82) remained unaltered. An overview of all biopsy data is presented in Table 1.

Discussion

The main objective of the present study was to assess whether local blood supply in exercising locomotory muscles is reduced after 8 weeks of local muscle unloading and if so, whether such an impaired blood supply would affect WoRPD in response to an incremental isokinetic exercise test. In

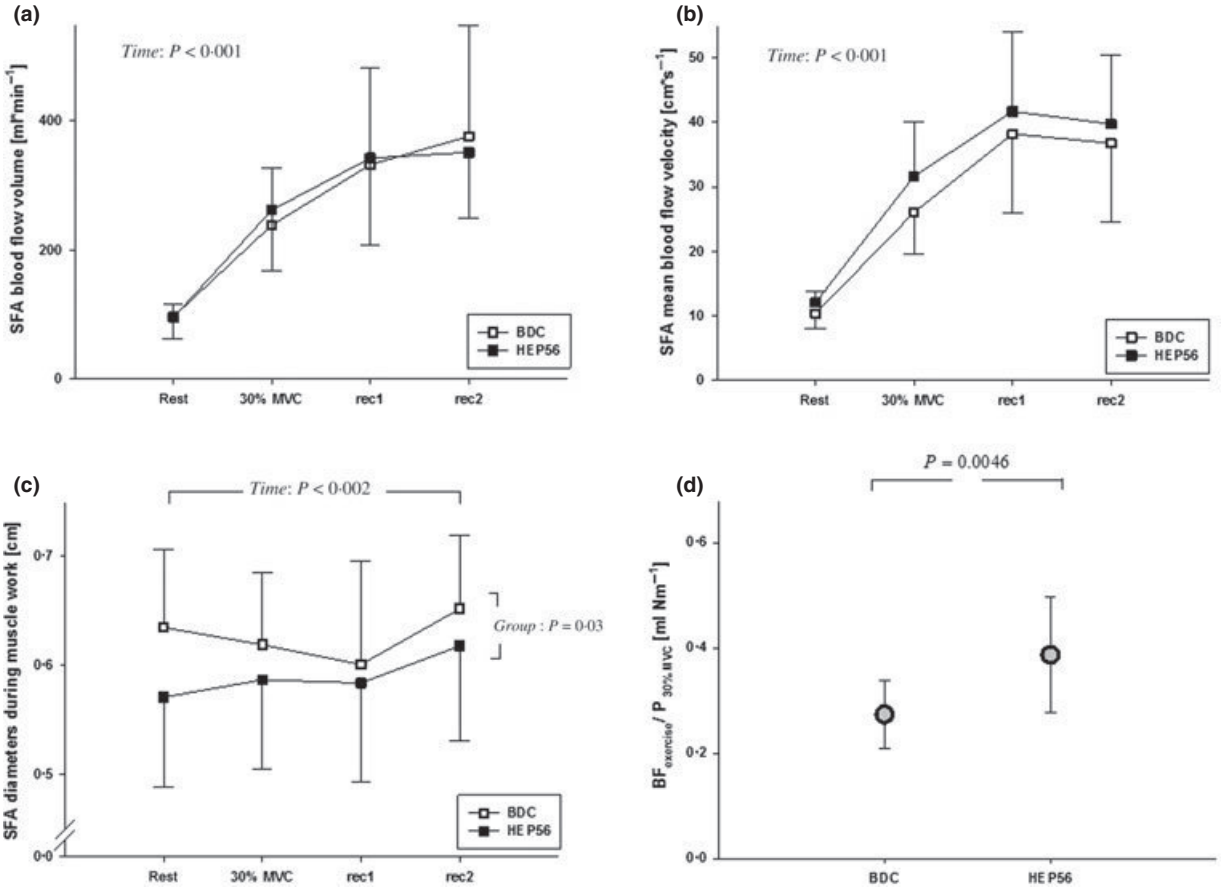

Figure 5 Arterial blood flow. (a) Absolute blood flow was not different between BDC and HEP56 for all conditions (P = 0·95). Within the IIET, blood flow, mean flow velocity (b) and SFA diameters (c) increased significantly over time and absolute arterial diameters were significantly smaller at HEP56 (P = 0·03). Panel (d) shows that the ratio of mean blood flow at 30% MVC ($BF_{exercise}$) and the corresponding plantar flexor power was significantly (P = 0·0046) higher at HEP56.

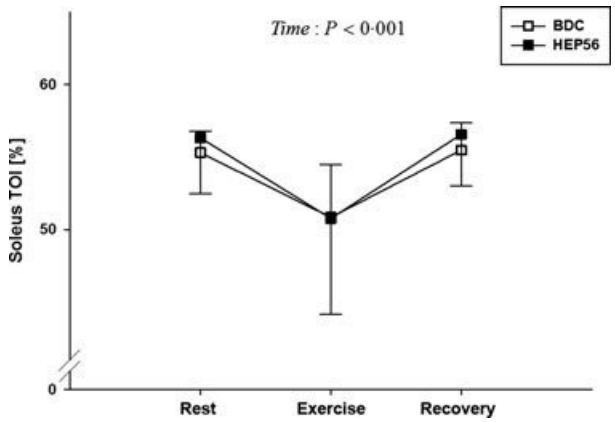

Figure 6 Soleus muscle tissue oxygenation. Soleus muscle TOI did not change significantly after the intervention (P = 0·65). In response to the IIET soleus muscle, TOI decreased significantly (P<0·001) from 55·8% (SD = 2·9%) at rest to 50·8% (SD = 5·2%) during exercise.

contrast to our expectations and in contrast to previous observations (Mulder *et al.*, 2007), the results of this study suggest that local arterial exercise blood flow in the atrophied soleus muscle was maintained after 8 weeks of muscle disuse. Furthermore, despite the slight reduction of capillaries around fibres, local tissue oxygenation, as assessed by near-infrared spectroscopy did not change nor was the intrinsic WoRPD of the plantar flexor muscle group affected by 8 weeks unloading.

Muscle performance

Maximal voluntary plantar flexor torque decreased significantly after the intervention. The 23·4% loss of maximal plantar flexor torque at HEP56 is greater than what can be attributed to mere atrophy of soleus muscle fibres, which seems to be a generic finding of unloading studies (Zange *et al.*, 1997; Alkner & Tesch, 2004; Mulder *et al.*, 2006). However, WoRPD, expressed as the relative power difference from MVC1–MVC5 to MVC_R throughout the IIET protocol (Fig. 4c), remained unaltered. Moreover, lactate concentrations that can be used as an indication for exercise-induced muscle fatigue (Finsterer, 2012) increased equally at BDC and at HEP56, reinforcing notion of an unchanged fatigability after the intervention. The concomitantly obtained EMG recordings also support this notion, as subjects did not show typical electrophysiological symptoms of increased muscle fatigue

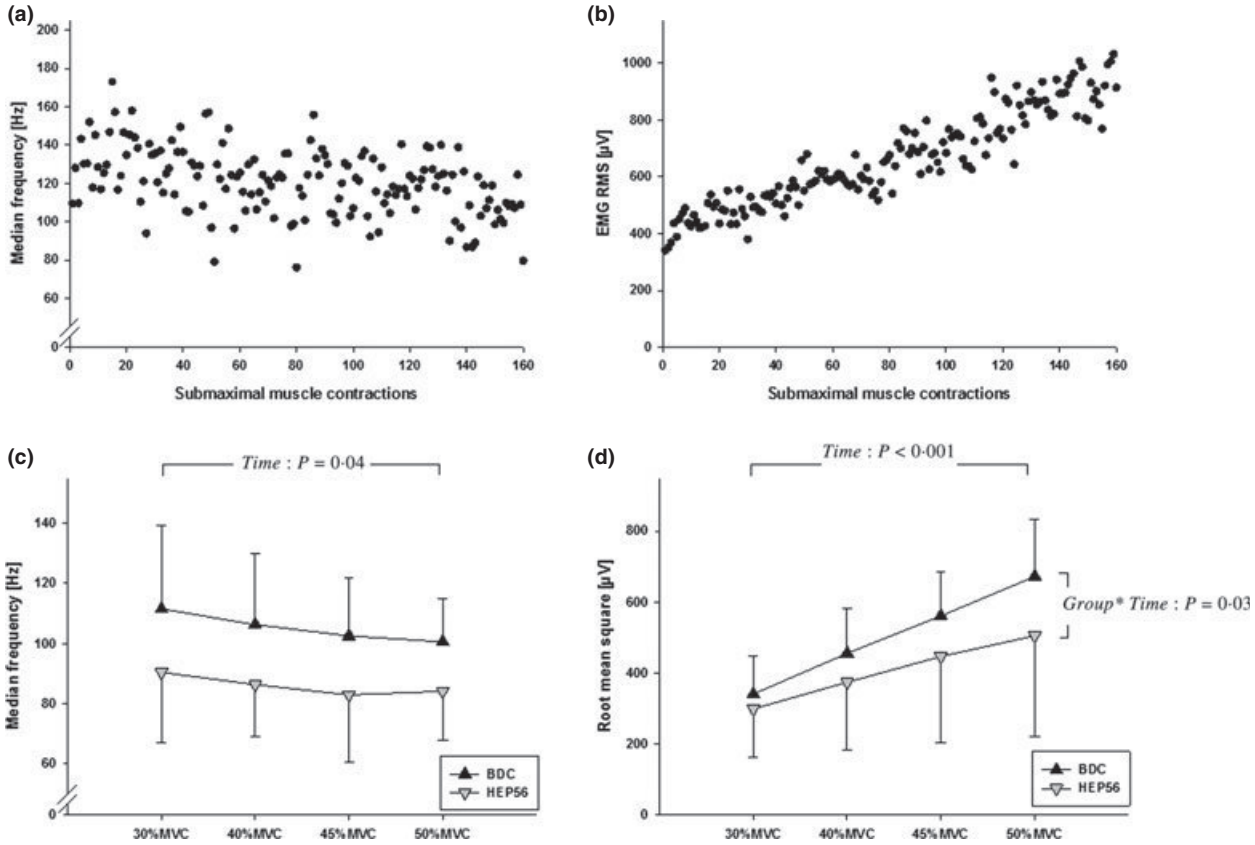

Figure 7 Soleus muscle electromyography. Shown are MF and RMS values that were calculated for each of the 160 submaximal plantar flexions. Panels (a) and (b) depict exemplary MF and RMS values for one subject. Panels (c) and (d) depict mean MF and RMS values for each stage. MF decreased significantly from stage to stage (P = 0·04) while the significant increase of RMS from stage to stage appeared to be more pronounced in response to the BDC IIET (P = 0·03).

Table 1 Soleus muscle morphology.

	Overall	Type I	Type II
DiaMin pre [μm]	71 ± 13	68 ± 11	75 ± 15
DiaMin post [μm]	65 ± 8·5	61 ± 7·7	69 ± 7·5
P-value	0·016	0·031	0·21
FCSA pre [μm^2]	14592 ± 5412	12939 ± 4955	16244 ± 5562
FCSA post [μm^2]	12381 ± 3642	10202 ± 2790	14561 ± 3101
P-value	0·06	0·06	0·40
CaF pre	4·2 ± 1·3	4·2 ± 1·3	4·2 ± 1·4
CaF post	3·6 ± 0·6	3·6 ± 0·6	3·5 ± 0·6
P-value	0·023	0·15	0·10
CD [nC mm^{-2}]	289 ± 123	–	–
CD [nC mm^{-2}]	229 ± 54	–	–
P-value	0·16	–	–
C:F pre [nC/nF]	2 ± 0·7	–	–
C:F post [nC/nF]	1·9 ± 0·7	–	–
P-value	0·53	–	–
Fibre type distribution pre [%]	–	68·4 ± 21·9	31·6 ± 21·9
Fibre type distribution post [%]	–	68·7 ± 12	31·3 ± 12
P-value	–	0·96	0·96
FCSA distribution pre [%]	–	64 ± 22·9	36 ± 22·9
FCSA distribution post [%]	–	62·6 ± 13·7	37·4 ± 13·7
P-value	–	0·82	0·82

following disuse, which would be indicated by more distinct RMS increases and more pronounced MF decreases in response to exercise (Masuda *et al.*, 1999; Hunter & Enoka, 2003). On the contrary, the amplitude of soleus muscle EMG during the submaximal muscle contractions increased less steeply after the study, which indicates that even less central drive was needed during the post-HEPHAISTOS IIET (Mulder *et al.*, 2007). The trend that overall MFs appeared to be reduced after the study is in agreement with a previous study, where MFs of the vastus lateralis muscle were consistently reduced after 56 days of bed rest in response to an isometric incremental exercise test (Mulder *et al.*, 2009). The decrease in MF reflects most likely a reduction of muscle FCSA as thinner muscle fibres, if compared to thicker fibres, have a reduced conduction velocity (Blijham *et al.*, 2006) that is accompanied by a reduced initial median frequency of the EMG power spectrum (Arendt-Nielsen & Mills, 1985). This endorses the morphological finding of the present study that soleus muscle fibres atrophied after 56 days HEPHAISTOS unloading. It could be argued here that the HEPHAISTOS did not entirely unload calf muscles during gait and that therefore muscle atrophy occurred without inducing any changes of fibre-type distribution that can be observed in conditions associated with complete muscle silencing (Burnham *et al.*, 1997). However, this remains speculative as it is unknown to what degree muscles need to be silenced to evoke fibre-type transformations and it is also possible that 8 weeks of muscle unloading were simply too short to induce such a change (Burnham *et al.*, 1997). Accordingly, the absence of a fibre-type transformation towards glycolytic type II fibres might have contributed to an unchanged WoRPD after the intervention.

Arterial structure and function

The structural and functional artery adaptations at rest, following 8 weeks of HEPHAISTOS unloading have been published elsewhere (Weber *et al.*, 2013). Those data revealed that resting SFA blood flow did not change after the intervention, despite an average 12·7% (SD = 6·6%) decrease in SFA calibres at rest. This observation is in corroboration with previous unloading studies (De Groot *et al.*, 2004; Bleeker *et al.*, 2005a, b). However, the focus of the present study was on blood flow during exercise, and to the best of our knowledge, there are no disuse studies available to date, that investigated this. The presented data show that absolute arterial exercising blood flow remained unaltered after 56 days of HEPHAISTOS unloading. In fact, the peak SFA blood flow (BF_{rec2}) was equally increased from resting conditions to 364 ml min^{-1} (SD = 139 ml min^{-1}) before and after the 56 days of unloading. As SFA diameters were significantly smaller at HEP56, an unchanged blood flow must have been compensated by an increased V_{mean}. At least visually, the data depicted in Fig. 4b seem to corroborate that V_{mean} is consistently greater at HEP56 when compared with BDC. However, statistically, this difference failed to reach significance. Of note, the postexercise dilation of 5·8% is in accordance with the magnitude of flow-mediated dilation (FMD) that was measured in the same study (Weber *et al.*, 2013). The latter finding suggests that fatiguing, although submaximal exercise does not cause maximal vasodilation of conduit arteries to supply working muscles with blood, as previous studies showed that the FMD response does not represent maximal dilation capacity (Bleeker *et al.*, 2005b). Considering the above, it seems to be plausible that the unchanged WoRPD can be attributed to the unchanged arterial blood flow, as previous studies related exercise-induced muscle fatigability to mainly resynthesis of phosphocreatine (PCr) that was found to be strongly linked to muscle blood flow (Zange *et al.*, 2008). However, it needs to be stated here that calf muscles constitute only a comparable small muscle mass and it could be argued if blood flow changes might occur when larger muscle volumes are involved. The finding that the arterial diameter did apparently not reach its maximal dilation capacity during the IIET might thus also attributed to the relatively small volume of the working muscles.

Tissue oxygenation and blood supply

During muscle work, the tissue oxygenation index (TOI) represents a dynamic balance of oxygen consumption and oxygen delivery (Boushel & Piantadosi, 2000). The presented NIRS data reveal that soleus muscle tissue oxygenation was similar at BDC and HEP56. This finding suggests that blood supply to working muscles was not compromised at HEP56, as one would expect greater oxygen desaturation in poorly perfused muscles (Mulder *et al.*, 2007). The latter is reinforced by the discovery that C/F was unaffected, because the same diffusion area for oxygen was available after the study. The fact that fibre-type distribution did not change after the 56-day intervention is also in agreement with the unchanged oxygen desaturation during muscle work, as oxygen consumption is dependent on oxidative capacity which in turn is thought to be largely dependent on fibre types (Takekura & Yoshioka, 1987). Albeit the marginal disadvantageous reduction of CaF and with regard to the atrophy of type I fibres, oxygen delivery to the working muscle might even have improved after the intervention as diffusion distances from capillaries to muscle mitochondria should have decreased. Nonetheless, the finding that blood lactate concentrations were similar between experiments, although absolute muscle work was reduced at HEP56, could indicate that the atrophied muscles relied more on glycolysis.

The ratio of blood flow (as measured during the first submaximal stage) and mechanical power (Fig. 5d) suggests a surplus of arterial blood supply after the intervention. As a consequence, HEP56 TOI should be higher than BDC TOI as, with regard to the unchanged capillary-to-fibre ratio, muscle perfusion and therefore oxygen delivery should have been 'luxurious'. Yet, TOI appeared to be similar between BDC and HEP56, suggesting that the muscle was not able to utilize the additional oxygen supplied. It could thus be that the flow that

was going through the SFA did not entirely go through the capillary bed of the soleus muscle, indicating a greater arteriovenous shunt volume after the study. The present findings are somewhat different from what has been found in a previous bed rest study of the same duration (Mulder *et al.*, 2007), where the TOI and the 'blood flow index' as measured with NIRS under administration of indocyanine green were found to be greatly reduced. However, measurement site (soleus versus vastus lateralis), the utilized unloading models and the applied exercise protocols (isokinetic versus isometric intermittent) differed between studies, making it difficult to compare the results.

Furthermore, evidence suggests that reductions of circulating blood greatly contribute to an increased exercise-induced whole body muscle fatigability and a decreased O_2 uptake after periods of bed rest (Convertino, 1997). However, in these all-out exercise tests, exercise-induced muscle fatigability is not normalized for losses of strength or muscle volume. In the present study, we normalized local exercise-induced muscle fatigability for losses of strength, and our data show that after local muscle unloading with HEPHAISTOS where muscles are greatly unloaded but not entirely silenced, blood flow to working muscles is not hindered. On the contrary, blood flow during and after exercise appears to be unaltered, suggesting a 'luxurious' conduit artery blood flow after the intervention. This might imply that peripheral vascular adaptations do not account for the disuse-induced reduction of VO_2 as seen in bed rest, at least during the first 8 weeks.

Conclusion

The presented results reveal that although maximal plantar flexor strength, soleus muscle fibre size and arterial dimensions decreased significantly, exercising blood flow and tissue oxygenation in the soleus muscle were maintained after 56 days disuse, and even increased when expressed in relative terms. Moreover, and possibly as a consequence of this, the presented data show that the soleus work-related power decrease, as a measure for exercise-induced muscle fatigue, following submaximal muscle work does not change after 56 days of local muscle unloading with HEPHAISTOS, if normalized to maximal muscle strength. The unchanged exercise-induced muscle fatigue is also reflected in the electromyographic activity of the soleus muscle where typical neuronal signs of muscle fatigue were not deteriorated. In a nutshell, the presented data suggest that the actual endurance quality of unloaded soleus muscle tissue does not change and that blood flow and oxygenation in working muscles do not constitute a limiting factor for ongoing submaximal muscle work after 56 days of local muscle unloading.

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Conflict of interest

The authors have no conflict of interests.

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Vascular adaptations induced by 6 weeks WBV resistance exercise training

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Summary

Background: The impact of whole-body vibration (WBV) upon the cardiovascular system is receiving increasing attention. Despite numerous studies addressing the acute cardiovascular effects of WBV training, very little is known regarding long-term adaptations in healthy humans.

Methods: A 6-week training study, with a 70 days follow-up was designed to compare resistive exercise with or without super-imposed whole-body vibrations. Arterial diameter, intima media thickness and flow-mediated dilation (FMD) were assessed by ultrasonography in the superficial femoral artery (SFA), the brachial (BA) and the carotid arteries (CA).

Results: SFA resting diameter was increased from 6·22 mm (SD = 0·69 mm) at baseline to 6·52 mm (SD = 0·74 mm) at the end of the training period ($P = 0·03$) with no difference between groups ($P = 0·48$). Arterial wall thickness was significantly reduced by 4·3% (SD = 11%) in the CA only ($P = 0·04$). FMD was not affected by any of the interventions and in any of the investigated arteries.

Conclusion: To the best of our knowledge, this has been the first study to show that the superposition of vibration upon conventional resistance exercise does not have a specific effect upon long-term vascular adaptation in asymptomatic humans. Our findings seem to be at variance with the findings observed in a bed-rest setting. One possible explanation could be that the independently saturable effects of flow-mediated versus acceleration-related endothelial shear stresses on arterial structure and function differ between ambulatory and bed-rest conditions.

Introduction

It is generally accepted that vessels, including large arteries, can adapt their structure as well as their functioning in response to alterations in their environment. As such, chronic disuse (e.g. spinal cord injury or bed rest) induces reduction in arterial diameter (Bleeker *et al.*, 2005a,b; De Groot *et al.*, 2006) and alterations in the ability to dilate (Bleeker *et al.*, 2005a,b; De Groot *et al.*, 2006). On the other hand, it has been shown that exercises such as running, cycling or walking can improve arterial function and structure and are through these direct vascular conditioning effects able to modify cardiovascular risk (Thijssen *et al.*, 2010). Of note, regular physical exercise is associated with a reduction of vascular events (Jolliffe *et al.*, 2001; Billinger, 2010), which highlights the importance of the matter under discussion.

It is mostly held that arterial adaptations occur in response to changes in shear rate acting on the endothelial layer (Tuttle *et al.*, 2001; Laughlin *et al.*, 2008). As shear rate is dependent on flow velocity and vessel cross-sectional area, muscle work influences internal vessel shear rate directly through increases in blood flow.

Whole-body vibration (WBV) is a novel exercise modality that is receiving increasing attention (Rittweger, 2010). Among many other things, WBV is affecting the cardiovascular system acutely, leading to an increase in blood flow velocity and tissue perfusion (Kerschan-Schindl *et al.*, 2001; Mester *et al.*, 2006) that is parametrically depending upon the frequency and amplitude of vibration (Lythgo *et al.*, 2009). The increased blood flow is thought to be in direct proportion to the enhanced oxygen demand by the working musculature (Rittweger *et al.*, 2002). Initially, however, tissue oxygenation of the calf muscles

appears to be increased during WBV, indicative of a 'luxury' perfusion for the acutely working muscle (Rittweger *et al.*, 2010). As one possibility it has been suggested that this effect is driven by endothelial shear stress (Rittweger *et al.*, 2010). The latter is quite likely to increase when the vibration is in line with the vessel axis (Yue *et al.*, 2007).

Very little, however, is known regarding the long-term effects of vibration upon vascular adaptive processes. In a bed-rest setting, Bleeker *et al.* (2005b) found that WBV in combination with conventional resistive exercise maintained the diameter in the leg conduit arteries of the exercise group and thus attenuated the decrease that was observed in a control group during 56 days of bed rest. However, there was no group that performed WBV only, or resistive exercise only, and it was therefore impossible to determine the specific effects of vibration. That question was addressed in a follow-up study that included an additional 3rd group that performed resistive exercise without vibration (Belavy *et al.*, 2010). It was found that vibration did indeed have a beneficial effect upon the conduit arteries above that was not achieved by resistive exercise alone (van Duijnhoven *et al.*, 2010).

The question arising from these studies is, however, whether vibration would have a specific effect upon long-term vascular adaption in people who are not subjected to bed rest. As, to the best of our knowledge, there is yet no study available that addressed this question, and based upon the theoretical framework outlined earlier, we hypothesized that superposition of vibration upon conventional resistive exercise would enhance the increase in diameter and in vascular dilation capacity within the setting of a training study.

The aim of the present study was, therefore, to examine the effects of 6 weeks of resistive exercise training with and without WBV exposure upon structure and function of the human vasculature, as well as the time course of any such effect. As a tertiary study aim, we sought to determine the retention of training-induced changes in vascular structure and function following 70 days after cessation of the training programme. Given the training regime involved the leg musculature, the focus of our attention was the superficial femoral artery (SFA). The brachial artery (BA) and the carotid artery (CA) were additionally studied to assess any systemic effects of the training regime.

Methods

Study design and subjects

The effects of Vibration Exercise study (EVE study) were designed as a stratified, randomized two-group parallel design. Twenty-six healthy men (26 ± 4 years) were recruited as participants. Two matched groups with regard to their maximum vertical jump height as an indicator of neuromuscular fitness (Runge *et al.*, 2004) were formed. A coin was then tossed to determine which group would perform either resistive vibration exercise (RVE) or resistive exercise (RE) only. Table 1 presents the anthropometric data at baseline. All subjects had been examined by a medical doctor before study inclusion. Exclusion criteria were as follows: diabetes; any known cardiovascular disease or abnormality; smoking; participation in strength training during the past 6 months; or any regular medication. Written informed consent was obtained from all subjects before commencement of the study. The EVE study protocol was approved by the Ethics Committee of the Northern Rhine medical association (Ärztekammer Nordrhein) in Duesseldorf (a rural suburb of Cologne).

Procedures

Training protocol

Exercises were performed using a guided barbell (Hoist fitness, San Diego, USA) and a side alternating vibration plate (Galileo Fitness; Novotec Medical GmbH, Pforzheim, Germany). All participants were familiarized with the training and equipment before the first training session. Subsequently, the individual training load was determined at 80% of the 1-Repetition Maximum (1RM; 80% of the 1RM equals eight repetitions of squats; squats were used as a reference to determine the individual training load) using the method described by Baechle & Earle (2000). Briefly, the subjects were loaded with an estimated weight and were then asked to complete as many repetitions as possible. The initial training load was then adjusted to (i) a higher load if the subject completed more than eight repetitions of squats or (ii) to a lower load if the subject completed fewer than eight repetitions of squats. During the first 2 weeks of the training intervention, two training sessions per week were completed. From the third week until intervention end, training was performed three times per week. As a warm up, two sets of heel raises and squats, in alternating order, were performed using the barbell (approximately 15 kg) without additional weights. A metronome was used as time emitter. The amplitude for the vibration was set to 6 mm (peak to base

Table 1 Subject characteristics.

	RVE group (n = 13)	RE group (n = 13)	P-value
Age (years)	24·31 (± 3·28)	23·38 (± 1·39)	0·52
Body mass (kg)	74·7 (± 6·94)	75 (± 4·67)	0·08
Height (m)	1·79 (± 0·05)	1·79 (± 0·05)	0·31
BMI	23·46 (± 2·1)	23·38 (± 1·4)	0·11
Systolic blood pressure (mmHg)	121 (± 4)	127 (± 8)	0·15
Diastolic blood pressure (mmHg)	71 (± 6)	72 (± 9)	0·89
Heart rate (beats/min)	57 (± 8)	55 (± 9)	0·70
Vertical jump height (cm)	41·7 (± 2·2)	42·2 (± 4·6)	0·97
BDC SFA diameters (mm)	6·30 (± 0·7)	6·14 (± 0·67)	0·54
BDC SFA IMT (μm)	366 (± 52)	331 (± 60)	0·13
BDC CA IMT (μm)	411 (± 50)	453 (± 58)	0·07

displacement) by the position of the feet on the plate. For the last set, the subjects should perform as many repetitions as possible. The individual load was recalculated after every training session applying the 1RM-method (Baechle & Earle, 2000) and using the last set of squats as a reference. The vibration plate was centred under the guided barbell, to allow optimal exercise performance. The RE group performed the exercises while standing on the same vibration plate, but without vibration stimulus. Each training session was supervised by an exercise scientist and took approximately 9 min. Blood pressure and heart rate were measured during each break. The protocol for the training sessions, as well as the exercise progression scheme, is depicted in Fig. 1.

Ultrasound measurements

Arterial diameters, intima media thickness (IMT) and flow-mediated dilation (FMD) were examined at baseline, after 1 week of training, after 3 weeks of training, after 6 weeks of training and 3 months after the last training session (BDC, EVE7, EVE21, EVE42 and follow-up, respectively). IMT was measured in the SFA and in the CA, while baseline diameters, blood cell velocity and FMD were measured in the SFA and in the BA. Blood cell velocity and resting diameter measurements of the BA were performed using an echo Doppler device (Mylab25; esaote, Firenze, Italy) with a 12–18 MHz broadband linear transducer. Blood cell velocity and resting diameter measurements of the SFA were taken with a 7·5–12 MHz broadband linear transducer. Anatomical landmarks such as the upper patella edge (for the SFA) and the radius epiphysis (for the BA) were recorded for all arteries to ascertain reproducibility of probe placement. Continuous measurement of velocity and diameter were taken using duplex ultrasound. For resting diameter measurements, videos with duration ≥ 1 min were recorded for offline analysis. For FMD assessment of SFA and BA, a cuff was placed distal to the probe that was inflated to 300 mmHg for 5 min. Ten second prior to cuff deflation video recording was started, and the FMD response was recorded for 5 min after cuff deflation. All videos were recorded on an external computer, using the analogue output of the device with a video grabbing system (GrabsterAV 450MX; Terratec, Nettetal, Germany) and an analogue to digital transformation software (MAGIX; Terratec).

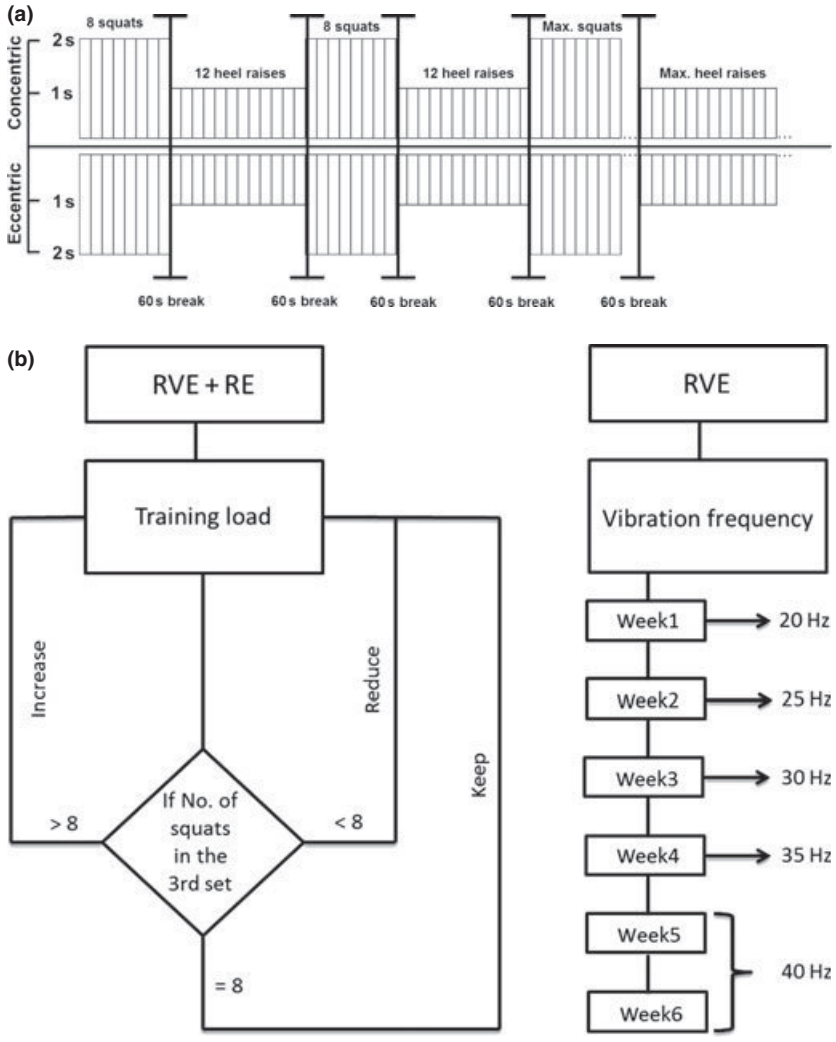

Figure 1 Exercise modalities. (a) Composition of one training session after warm up. The subjects accomplished three sets per exercise, with a 60 s break in-between the sets. In the last set of each exercise, the subjects were asked to perform as many repetitions as possible. (b) The individual load was recalculated after every training session using the 1 RM method with the last set of squats as a reference. The vibration stimulus for subjects in the RVE group was weekly increased with 5 Hz from 20 Hz to maximally 40 Hz during weeks 5 and 6.

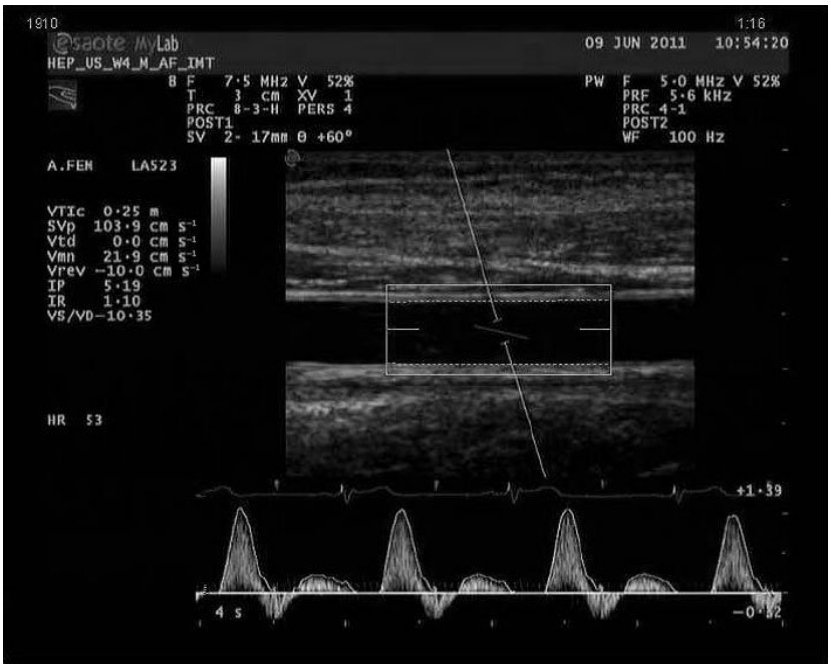

Figure 2 Off-line video analysis. Diameters within the ROI were measured continuously using the 'Vasculometer'- edge detection (Bremser *et al.*, 2012) software and a sampling frequency of 25Hz.

All measurements were taken at the same time of the day to avoid circadian variation. Prior to the measurements, subjects rested in a darkened room for at least 20 min in supine posture. Subjects fastened prior to the measurements for ≥ 8 h and refrained from caffeine, alcohol and exercise for ≥ 8 h before the measurement.

Data processing

Intima media thickness

The IMT was determined by the IMT software tool (esaote, QIMT, for MyLab25). The IMT analysis tool processes the radio frequency signal (RF-signal) from the ultrasound device in real time. IMT videos were recorded for ≥ 5 heart cycles, using a 7·5–12 MHz broadband transducer placed parallel to the assessed artery. The region of interest (ROI) for IMT measurement was placed at the region of the artery with the highest image quality. IMT video analysis was performed using a video sequence of ≥ 5 heart cycles. The IMT videos were analysed offline, and the value with the lowest standard deviation (≤ 20 μm) was taken as IMT. Blood pressure was measured at five time points before the first measurement and during each cuff inflation/deflation period, using an electronic sphygmomanometer (medicus pc, boso, Jungingen, Germany). Heart rate was measured continuously using the internal 3-lead ECG of the ultrasound device.

Diameters and flow-mediated dilation

All videos were analysed off-line. Duplex video analysis was performed using a custom-produced edge detection and wall tracking software (Fig.2) Vasculometer 1.2, (Bremser *et al.*, 2012). The signal from the wall-tracking software was processed with MATLAB (Mathworks, Natick, MA, USA), using a moving average filter with a span of 500 frames. The median of all processed values before cuff deflation was taken as resting diameter. The highest value of the filtered signal was identified and used as peak diameter after cuff release. The FMD response was then expressed as the relative increase in diameter after cuff deflation (see Fig. 3).

Statistical analysis

Statistical analyses were performed using STATISTICA 8.0 for Windows (Statsoft, Tulsa, OK, USA, 1984–2008). A Repeated

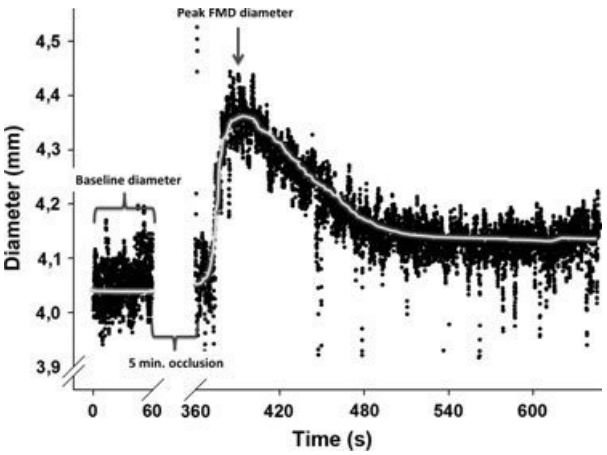

Figure 3 Signal Processing for FMD analysis. The dark signal depicts the raw signal of the brachial artery diameter of one subject as measured with the automated wall detection software. The white line plots the signal, filtered with a moving average filter (span: 500 frames). The diameter was not recorded during the occlusion. All measurements were taken with a sampling frequency of 25 Hz.

measures ANOVA was performed with time (five different points) and group (RVE versus RE) as main factors, as well as an interaction between main factors. A repeated measures ANCOVA was performed to assess the effect of training load progression as a covariate on SFA baseline diameters. Values are given as means ± SD. There were 10 missing values out of 156 in the RE group and 12 out of 156 in the RVE group. Those values were linearly interpolated using adjacent data. Tukey's test was used for post hoc testing. Differences regarding the anthropometric characteristics at baseline were assessed performing a non-paired t-test.

Results

The subject's anthropometric characteristics, as well as maximum countermovement jump height, were comparable between groups (see Table 1), and anthropometric characteristics were unchanged during the course of the study. Because of medical reasons, two of the initially 15 starting RVE subjects were not able to complete the intervention. One subject had to quit the study after 2 weeks because an acute back injury induced by the training, the other subject dropped out after the 4th intervention week because of exercise-related headache. It was not possible for the subjects of the RVE group to reach maximal plantarflexion during the heel raise exercise; however, this 'handicap' was not noted for the subjects of the RE group.

Training progression

From the beginning of the intervention, both groups showed an almost linear increase in the training loads (see Fig. 4). Compared to the first training session, the increase in training loads during the 6 weeks intervention reached 46·9% (SD = 18·9%) in the RVE group, which was less than the increase in training loads by 59·8% (SD = 17·3%) observed in the RE group (time*intervention: P<0·001).

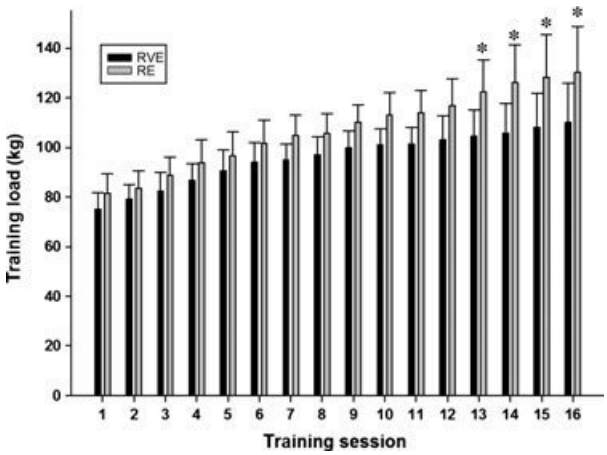

Figure 4 Increase in training loads. The individual load was recalculated after every training session using the 1RM method with the last set of squats as a reference. The mean training load of the RE group was higher throughout the whole intervention. The difference between the groups reached significance after the 13th training session (time: P<0·001; time*intervention: P<0·001).

Arterial diameter

All structural and functional parameters are illustrated in Fig. 5. Compared to baseline data collection (BDC), SFA resting diameter increased in both groups by 7·2% from the 3rd training week (EVE21) onwards (P<0·001), after which time there was no further increase observed (P = 0·58; see Fig. 5a). There was a tendency (P = 0·06) that the diameter was still increased after 70 days following training, comparing follow-up and BDC diameters (see Fig. 6). The resting diameter of the BA was not affected by any of the interventions, however, a tendency (time: P = 0·06) reveals a slight systemic adaptation of the BA over time for both groups (see Fig. 5b). We did not detect any significant difference between the two interventions regarding the time course and the magnitude of the diameter adaptations for both SFA (time*intervention: P = 0·96) and BA (time*intervention: P = 0·20).

Intima media thickness

No effect of time (P = 0·42) or intervention (P = 0·11) was observed for the IMT of the SFA (see Fig. 5c). Intima Media Thickness of the CA was significantly lower (−4·2%) after the intervention, but no difference was observed between the two groups (time: P = 0·04; time*intervention: P = 0·11; see Fig. 5d).

Flow-mediated dilation

No effects of time or intervention were observed for the FMD response of the SFA (time: P = 0·45; time*intervention: P = 0·60; see Fig. 5e) or the BA (time: P = 0·27; time*intervention: P = 0·99; see Fig. 5f). Furthermore, the time to peak FMD for both the SFA (time: P = 0·46; time*intervention: P = 0·25) and the BA remained unaltered for both groups throughout the experiment (time: P = 0·44; time*intervention: P = 0·15).

Discussion

The main aim of this study was to investigate the specific effects that whole-body vibration might have in a training setting upon structure and function of conduit arteries in healthy ambulatory subjects. The results suggest that RE training and RVE training equally lead to increases in resting diameter of the SFA. This finding is in line with the existing literature, in that it supports the notion of training-induced enlargement of arterial diameter (Naylor *et al.*, 2006; Thijssen *et al.*, 2010; Rowley *et al.*, 2011). However, in contrast to our expectations, there was no difference between the two intervention groups in any of the parameters tested in the present study. Moreover, no effect by the training intervention was

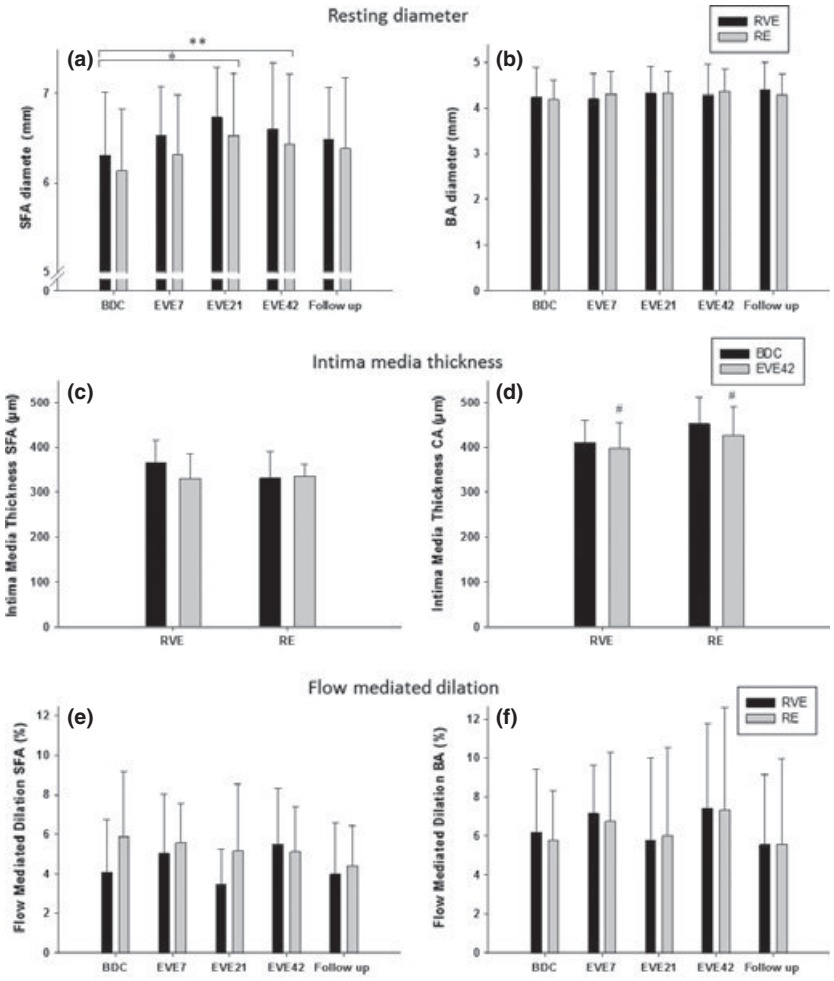

Figure 5 Arterial parameters. Panel a depicts the time course of SFA resting diameter throughout the study for both RE and RVE. No significant differences existed between the responses of both groups. Across groups, SFA diameter significantly increased from EVE21 onwards by 6·6% (**P = 0·003, *P<0·001). Panels b and c show, respectively, that there were no changes in BA diameter and SFA IMT. Panel d shows that CA IMT was significantly reduced after the intervention ($^{\#}$P = 0·04). Panels e and f show that the FMD remained unaffected for both SFA and BA.

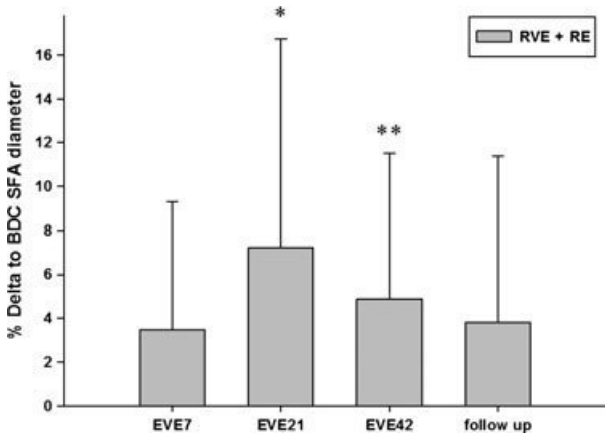

Figure 6 SFA resting diameter adaptation. Relative values of the SFA resting diameter increases, using the pooled data of both intervention groups. Resting diameters are significantly increased for EVE21 (*P<0·001) and EVE42 (**P = 0·003) compared to the BDC values. There is a tendency that the diameters are still increased 70 days after the intervention, comparing follow-up and BDC values (P = 0·06).

elicited upon flow-mediated dilation or IMT. Both of these observations are in stark contrast to findings in a bed-rest setting (Bleeker *et al.*, 2005b; van Duijnhoven *et al.*, 2010).

Changes of SFA resting diameter

Our main hypothesis was based upon the view that (i) endothelial shear stress is a main driver in adaptation of resting diameter in the conduit arteries and that (ii) vibration exercise will enhance endothelial shear stress. It is our opinion that the current data, although collected meticulously and with great care, do not provide strong enough evidence to put the first assumption into question because of the bulk of literature in their support (Langille & O'Donnell, 1986; Tuttle *et al.*, 2001; Balligand *et al.*, 2009). In many biological systems, however, the frequency composition of a controlling signal is of great importance, and it seems that considering the frequency composition of shear stress imposed by arterial (= pulsatile) flow could be relevant, too. In the light of this consideration, the obvious conclusion from the present study would be that it could be flow-mediated, rather than acceleration-induced shear stress that matters for the adjustment of resting diameter. On the other hand, this explanation would be at variance with the aforementioned findings in the bed-rest setting (Bleeker *et al.*, 2005b; van Duijnhoven *et al.*, 2010). Importantly, there is no substantial metabolic demand upon the immobilized leg musculature, and flow-mediated shear

stresses must therefore be expected to be low. In this 'bradytrophic' situation, the provision of resistive exercise might engender increased blood flow in the absence of any substantial acceleration-induced shear strains in the conduit arteries. Of note, walking and running is associated with vertical accelerations of up to 10 g (Lafortune, 1991), meaning that the habitual activity in an ambulatory setting is indeed likely to provide acceleration-related endothelial shear stresses. One way of reconciling findings from this study with those from the aforementioned bed-rest studies, therefore, would be in assuming that acceleration-induced shear stress does have an effect upon resting diameter that is independent of flow-mediated shear stress and that his effect saturates under ambulatory conditions with habitual activities. Although the training loads differed significantly between RVE and RE group for the last four training sessions, training load progression did not have any measurable effect on resting SFA diameters, as yielded by ANCOVA ($P = 0{\cdot}17$, data not shown).

Time course of adaptive changes and their retention

Previous studies showed that functional adaptations occur rapidly after the onset of an exercise intervention and precede structural adaptations (Haram *et al.*, 2006; Tinken *et al.*, 2008). However, we did not detect any interplay between functional and structural adaptations, regarding their time course. An interesting finding of the present study is in relation to the time course of vascular adaptation: the change in SFA resting diameter has reached its maximum after the 3rd training week (EVE21) and did not further increase during the subsequent 3 training weeks. The steady state in luminal expansion of the SFA could not be overcome by the progressive increase in training loads by increasing weight and vibration frequency (the latter RVE only) during the study. One way of explanation would be that a functional state had been achieved at EVE21 that did not necessitate any further increase in flow capacity to accommodate the increase in exercise-related energy expenditure. In this context, it is useful to consider that the flow capacity of skeletal muscle is by far greater than could be covered by the cardiac output (Andersen & Saltin, 1985).

Investigations of the retention of training effects provide helpful information about the preventive quality of a training regime. To date, only few studies investigated the retention of training interventions regarding vascular adaptation effects. We found that the BDC resting diameter of the SFA was not statistically different compared to the diameter measured at the follow-up session 70 days after intervention end. However, the difference between the BDC resting diameter and the diameter measured 90 days after the intervention failed to reach statistical significance only by a small amount ($P = 0{\cdot}06$), revealing the possibility that some structural adaptation might have been maintained beyond the end of the intervention and just faded away before the follow-up testing (see Fig. 6).

Intima media thickness

Intima media thickness, as measured with B-mode ultrasound, provides an index of sub-intimal thickening. IMT of the carotid artery, for example, is commonly used as a surrogate marker for preclinical atherosclerosis and is strongly related to cardiovascular risk factors and diseases (Thijssen *et al.*, 2012). Previous studies reported no or only a modest impact of exercise interventions on carotid artery IMT (Tanaka *et al.*, 2002; Rakobowchuk *et al.*, 2005). However, in the present study, both intervention groups showed a significantly reduced IMT of the CA. Thijssen *et al.* (2012) recently concluded that high exercise intensities or high exercise volumes are required to affect carotid artery IMT. Indeed, the present study does not satisfy the latter aspect because the subjects exercised only for approx. 30 min week^{-1}, but as they trained with very heavy loads (80% MVC), one could commonly regard the present training regime as 'intense'. Furthermore, the effects of exercise interventions on the IMT of peripheral arteries that supply the exercising muscles are thought to be more pronounced than in the CA (Moreau *et al.*, 2002). Nonetheless, there were only significant changes in the CA but not in the SFA in the present study. One explanation for this finding could be that the mean SFA IMT of all subjects (348 μm, SD = 57 μm) was already too low to detect further decrease adaptations, whereas the mean CA IMT (432 μm, SD = 57 μm) still had some 'buffer' to further adapt downwards.

Flow-mediated dilation

Physical exercise is thought to improve FMD, a measure of endothelial function. In this context, blood flow-induced shear stress acting on the endothelial layer seems to be the main driver. As different exercise regimen lead to different blood flow patterns (Thijssen *et al.*, 2009) and different exercise intensities lead to acute changes in the bioavailability of Nitric Oxide (NO) (Goto *et al.*, 2003), previous training interventions showed heterogeneous findings regarding their impact upon vascular function. Goto *et al.* (2003), for instance, showed in their study that only training at moderate intensities (50% VO_2-Max) was able to affect FMD. This would explain the findings from the present study, whereas FMD was not affected by any of the two interventions. Furthermore, the present results confirm earlier findings in healthy subjects (Rakobowchuk *et al.*, 2005; Thijssen *et al.*, 2007) and suggest that arterial function is more prone to enhance in patients (Moriguchi *et al.*, 2005; Andreozzi *et al.*, 2007) than in healthy individuals. However, our findings remain discordant with the findings observed in a bed-rest setting that showed that only RVE was able to attenuate the immobilization induced increase of FMD (Bleeker *et al.*, 2005b; van Duijnhoven *et al.*, 2010), while RE failed to impact FMD. Though, the mechanisms responsible for the altered hemodynamic situation during WBV are currently unclear. Both an increased metabolic demand of the working muscles during

WBV (Rittweger *et al.*, 2002), and the arterial wall accelerated with the other leg tissues around the 'inert' blood column might constitute a crosstalk of shear stress trigger signals. A separated analysis of the arterial blood flow during passive vibration, during RE alone and during RVE, admittedly a very challenging approach, would provide helpful information to complete our picture about the hemodynamic situation during WBV.

Conclusion

In conclusion, 6 weeks of resistive training for three times per week led to significant adaptations of the SFA diameter regardless of whether it was combined with whole-body vibration or not. No intervention had an effect on arterial function. We did not observe local effects, but we observed systemic effects concerning changes in wall thickness. These findings seem to be at variance with findings in bed rest. Of note, the subjects being investigated in this study were healthy subjects. A similar study design applied to a diseased population with poor vascular structure and function might be more in line with the findings observed under bed-rest conditions. One possible explanation could be that the independently saturable effects of flow-mediated versus acceleration-related endothelial shear stresses on arterial structure and function differ between ambulatory and bed-rest conditions. However, RVE training as conducted in the present study is highly demanding and exhaustive for the subjects and exercise parameters would therefore have to be tailored for people in a diseased state.

Perspective

As WBV is an exercise modality that is gaining more and more popularity across all kinds of gyms, we wanted to investigate its effects upon arterial structure and function in healthy ambulatory subjects. Our data suggest that resistive exercise leads to both an increase in the resting diameter of the femoral artery and a decrease in the carotid artery wall thickness, and that these effects are not enhanced by super-imposed vibration. The underlying acute hemodynamic situation during bouts of vibration exercise, however, needs to be further investigated to better understand the influence of different blood flow patterns and to explore the impact of gravity-driven, acceleration-related shear stress, acting on the endothelial layer.

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Conflicts of interest

The authors have no conflicts of interest.

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5.3 Curriculum Vitae

Tobias Weber was born on the 30th October 1982 in Prüm, Eifel, Rhineland-Palatinate, Germany. After having finished High School, receiving the German degree 'Abitur' in 2002, he started working in a residential accommodation for mentally handicapped people performing his Civilian Service. In 2003 he started studying Exercise Science at the German Sport University in Cologne. During this time he gained insights into various life science topics and experienced also the unique summer of 2006 when Germany hosted the football world cup. He finished his study at the Sport University in 2008, writing the Diploma thesis entitled 'The influence of high altitude training on the senescence of endothelial progenitor cells' at the Department of Molecular and Cellular Sport Medicine, under the supervision of Prof. Dr. Wilhelm Bloch and Prof. Dr. Klara Brixius. In the year 2009, following a stay abroad in Southeast Asia, he took up his duties as a PhD candidate at the German Aerospace Center (DLR) Cologne, where he received a 'Space Life' scholarship which is funded in equal parts by the Helmholtz Association and the German Aerospace Center. During the time as a PhD candidate he worked in the Space Physiology Division led by Prof. Dr. Jörn Rittweger in corporation with the Department of Molecular and Cellular Sport Medicine of the German Sport University, led by Prof. Dr. Wilhelm Bloch. His research mainly comprises adaptations of the arterial system induced by muscle unloading and exercise training.