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ANGIOGENIC STIMULI OF RESISTANCE EXERCISE AND SUPERIMPOSED WHOLE-BODY VIBRATIONS

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DECLARATION / ERKLÄRUNG

Hierdurch versichere ich: Ich habe diese Arbeit selbständig und nur unter Benutzung der angegebenen Quellen und technischen Hilfen angefertigt; sie hat noch keiner anderen Stelle zur Prüfung vorgelegen. Wörtlich übernommene Textstellen, auch Einzelsätze oder Teile davon, sind als Zitate kenntlich gemacht worden.

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

Åsa Beijer, Köln, den 31.07.2013

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CONTENTS

1 Abstract

OBJECTIVES: Whole-body vibration (WBV) training has become a popular training mode in the past few years and is nowadays applied in various fields like sports, rehabilitation and preventive medicine. WBV training has been shown to improve peripheral perfusion and may elicit muscle deoxygenation. We hypothesized that the superposition of WBV to resistance exercise would add a pro-angiogenic stimulus to the training and we aimed to find a novel training mode that concurrently induces muscle hypertrophy and capillary growth in order to improve muscle performance. *METHODS:* A six-week training study including 26 healthy males was conducted in a randomized two-group parallel design, in which one group performed resistance exercise (RE) and the other group resistance exercise with superimposed whole-body vibrations (RVE). Subjects trained 2-3 times per week, concluding 16 training sessions. The training consisted of squatting exercise and calf raises performed with heavy loads that were set at 80% of the one-repetition maximum. During the initial and final exercise sessions of the 6-week intervention, measurements were performed at rest, during and acutely after exercise. Blood volume and tissue oxygenation were determined in gastrocnemius via near infrared spectroscopy. Angiogenic markers (matrix metalloproteinase -2 and -9, vascular endothelial growth factor (VEGF) and endostatin) were measured in serum via ELISA and the proliferative effect upon human umbilical vein endothelial cells was determined *in vitro*. Finally, long-term effects of the trainings on muscle morphology were determined in soleus biopsies. *RESULTS*: Our data are to our knowledge the first to describe transient increases of circulating angiogenic markers after resistance exercise. VEGF levels were acutely higher in the RE group, which supposedly provoked increased proliferation of endothelial cells *in vitro.* Furthermore, acute increases in circulating endostatin were higher in the RE group after the six-week training intervention. These effects were elusive in the RVE group. Despite differences in acute VEGF levels, capillary growth in soleus muscle was not different between groups. However, total blood volume and exercise hyperemia was increased after six weeks of RVE training.

CONCLUSIONS: Our data indicate the pro-angiogenic stimulus of RE is not increased by superimposing WBV to the training. While structural adaptations in muscle tissue were similar in both groups, regular RVE training seems to influence the functional state of small arterioles and potentially capillaries, enhancing muscle perfusion and post-exercise hyperemia.

2 Introduction

2.1 Skeletal muscle plasticity

Skeletal muscle is a plastic tissue that has unique abilities to adapt its structural properties to alterations in demand, such as exercise training, unloading and hypoxia [1]. Generally, skeletal muscle plasticity acts according to the principle 'form follows function', which is the essence of the law of nature described by Aristotle in 350 B.C. [2]. Phenotypic adaptations in skeletal muscle induced by physical exercise are determined by contraction mode, magnitude of loading, contraction duration, contraction velocity and the number of muscle contractions performed [3]. For example, a training stimulus inducing high-frequent repetitions with low training loads like endurance exercise will induce adaptations towards fatigue resistant muscles (i.e. increases in oxidative capacity and capillarity) [4], whereas resistance exercise performed with heavy loads and comparably few repetitions will induce adaptations in the trained muscles that enable increased force production via muscle hypertrophy [5]. It would be desirable to find a training mode that combines both, i.e. concurrently stimulating muscle hypertrophy and capillarization. The present thesis focuses on capillary growth induced by resistance training with and without superimposed whole-body vibrations as well as on acute and long-term effects upon skeletal muscle microcirculation. Hence, the following introduction will give insights into how muscle perfusion is regulated acutely during exercise and furthermore present an overview on angiogenic stimuli and factors inducing capillary growth in skeletal muscle.

2.2 Mechanical and metabolic stimuli inducing vascular adaptations

Resting skeletal muscle has a relatively low oxygen consumption and a poor perfusion rate, ranging from 1-4ml blood·min^{-1} per 100g muscle tissue [6]. When skeletal muscle transits from a resting to an exercising state, oxygen consumption can increase 20-50 fold, depending on intensity and duration of the exercise [7]. It is suggested that muscle contractions *per se* elevate tissue perfusion through the so-called 'muscle pump' function which increases the capillary-venous pressure gradient in the vascular bed of skeletal muscles [8]. However, perfusion induced by the muscle pump is not sufficient to meet the increased metabolic demand and oxygen consumption of active skeletal muscles. Hence, arteries and arterioles have the ability to dilate, resulting in exercise-induced increases in

muscle perfusion (*hyperemia*) in skeletal muscle, enabling perfusion rates of 50-100ml blood·min^{-1} per 100g muscle tissue [6,9]. This locally stimulated vasodilation results from the complex interplay between neural, mechanical and metabolic stimuli. Activity of the sympathetic nervous system is elevated during exercise [10,11], mediating alpha-adrenergic vasoconstriction (i.e. reduction in vessel diameter) in resistance arteries and arterioles of inactive skeletal muscles and peripheral organs like spleen and gastrointestinal tract [7]. This mechanism ensures redistribution of blood flow to active skeletal muscles [12–14], where systemic vasoconstriction is exceeded by locally stimulated vasodilation [9].

Mechanical forces derived during shortening and relaxation of myocytes exposes blood vessels to tensile forces, acting from the abluminal side on the vasculature [15]. Also, extravascular pressure increases during muscle contractions, causing a transient decrease in capillary radius [16]. Furthermore, exercise-induced dilation of arteries and arterioles results in increased blood cell velocity and capillary hydrostatic pressure within the microvasculature of active skeletal muscles. Increased blood cell velocity and / or a decreased capillary radius in turn leads to elevated shear stress (τ) which is acting on endothelial cells (ECs), according to the formula $\tau = \eta\ (4*V_{BC} / R)$, where τ is shear stress, η is blood viscosity, V_{BC} is blood cell velocity, and R is capillary radius. A decrease in vessel diameter and / or elevated hydrostatic pressure (P) increases capillary wall tension (T) following La Place's law T = P*R. Wall tension and shear stress are thought to be the basic hemodynamic forces acting on endothelial cells during exercise that induce vascular adaptations such as capillary growth, see Fig. 1 [17].

Moreover, elevated muscle activity leads to increases in metabolic rate and oxygen consumption, resulting in decreased partial oxygen pressure (PO_2) and hypoxia in muscle tissue [18]. Oxygen delivery to the tissue is essential since muscle cells have limited stores of oxygen and an enduring under-supply of oxygen causes tissue atrophy. The human body has developed multiple oxygen-sensitive mechanisms to induce vascular adaptations that are based upon hypoxia or ischemia, when metabolic rate exceeds oxygen delivery [19], see paragraph 2.4.2.2.

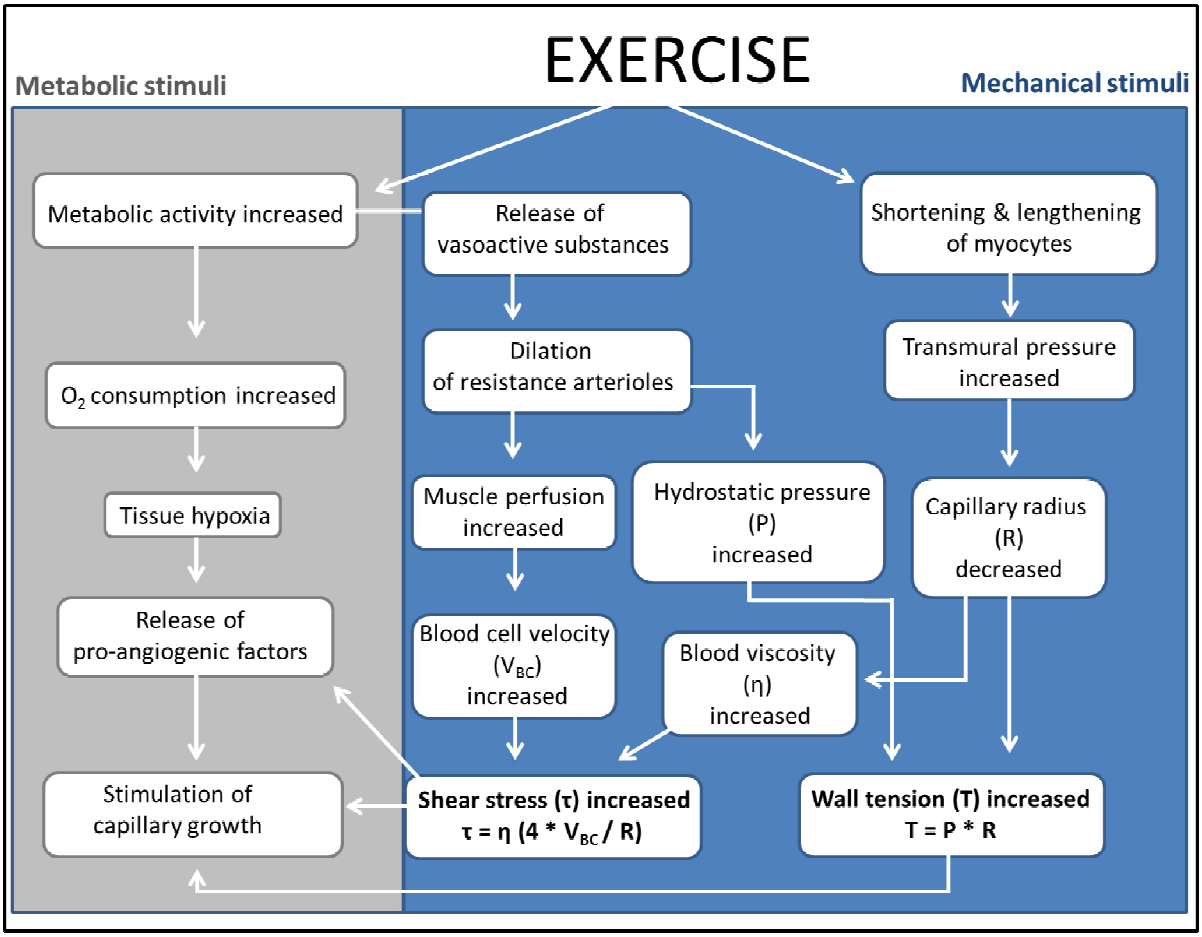

Figure 1. Summary of metabolic and mechanical stimuli arising from repeated muscle contractions during physical exercise. For more information see text above. According to Brown & Hudlicka, 2003 [17].

2.3 Mechanisms of exercise hyperemia

Skeletal muscle perfusion is determined by the contractile state of vascular smooth muscle (VSM) cells, which are localized in a circular manner around the endothelium of arteries and arterioles [20,21]. The mechanisms of VSM contraction and metabolites that induce VSM relaxation, i.e. dilation of resistance arteries and arterioles, will be introduced in the following paragraph.

2.3.1 Control of vascular smooth muscle contraction

The contractile state of VSM cells is determined by cytosolic free calcium (Ca^{2+}) concentrations [20]. Increased Ca^{2+} concentrations favor the formation of the Ca^{2+}-calmoduline (CaM) complex, which activates myosin light chain kinase (MLCK), which in turn phosphorylates regulatory sites on the myosin light chain, leading to the formation of cross-bridges between actin and myosin, thereby inducing vasoconstriction [22,23]. Myosin light

chain kinase action is opposed by myosin light chain phosphatase (MLCP), which de-phosphorylates the myosin light chain and induces vasorelaxation [20,21]. Key regulatory mechanisms leading to vasoconstriction or vasorelaxation act via modulating intracellular Ca^{2+} concentrations, Ca^{2+} sensitivity, or via modulation of MLCK and MLCP activity [24] and are regulated mainly via ion channels and via cyclic nucleotide signaling in VSM cells [25]. Potassium (K^+) channels modulate intracellular Ca^{2+} concentrations and play a central role in the control of microvascular tone. In VSM, opening of K^+ channels and K^+ outward flux leads to membrane hyperpolarisation and subsequent closure of voltage-driven Ca^{2+} channels, resulting in VSM contraction [26]. Main metabolites and mechanisms involved in locally stimulated vasorelaxation in exercise hyperemia are depicted in Fig. 2 and will be introduced in the following paragraph.

2.3.2 Vasoactive Substances

Muscle activity induces myocytes and ECs to produce vasoactive substances like prostaglandines, adenosine, nitric oxide (NO) [25,27] and lactate [28]. The latter is a metabolite produced by skeletal muscle upon anaerobic generation of adenosine triphosphate (ATP) [29]. These substances diffuse to the adjacent vascular smooth muscle cells and induce vasodilation: prostaglandines and adenosine activate adenylyl cyclase, whereas NO and lactate activate guanylyl cyclase [25]. This results in increases of intracellular levels of cyclic adenosine monophosphate (cAMP) or cyclic guanine monophosphate (cGMP), respectively. Subsequently, activation of downstream cGMP or cAMP-dependent effector kinases occurs, influencing MLCP and MLCK activity and cytosolic Ca^{2+} concentrations, thereby inducing vasorelaxation [24]. Increased cAMP or cGMP concentrations have furthermore been described to induce vasorelaxation via activation of K^+ channels, thereby causing membrane hyperpolarization [26]. The so-called endothelial derived hyperpolarization factor (EDHF) induces VSM hyperpolarization and vasodilation via yet not completely understood mechanisms [30], possibly involving K^+ channels [31].

In addition, neural stimulation of muscle contractions induces a spillover of the neurotransmitter acetylcholine (AcH) at neuromuscular endplates, which has also a vasorelaxing effect, possibly via NO release from ECs [32]. Furthermore, with every contraction/relaxation cycle, K^+ is lost to the extracellular space [33,34], inducing vasorelaxation [35,36]. Moreover, increased oxygen consumption in active skeletal muscle implicates decreased venous PO_2, which is thought to provoke ECs to release vasodilatory

factors [37]. Adenosine triphosphate is also considered being a potent vasodilator [38], which increases in blood and interstitial fluid during exercise [39,40]. It is thought that ATP is released by ECs [41,42], by sympathetic nerve terminals [41] and by red blood cells (RBC) in response to mechanical deformation [42]. Finally, ATP induces vasodilation via activation of P_{2Y} purinergic receptors on vascular ECs, inducing subsequent release of vasodilators such as NO, prostaglandins (PGs) and EDHF [43]. Of note, erythrocytes also influence the contraction state of arterioles via NO release [25].

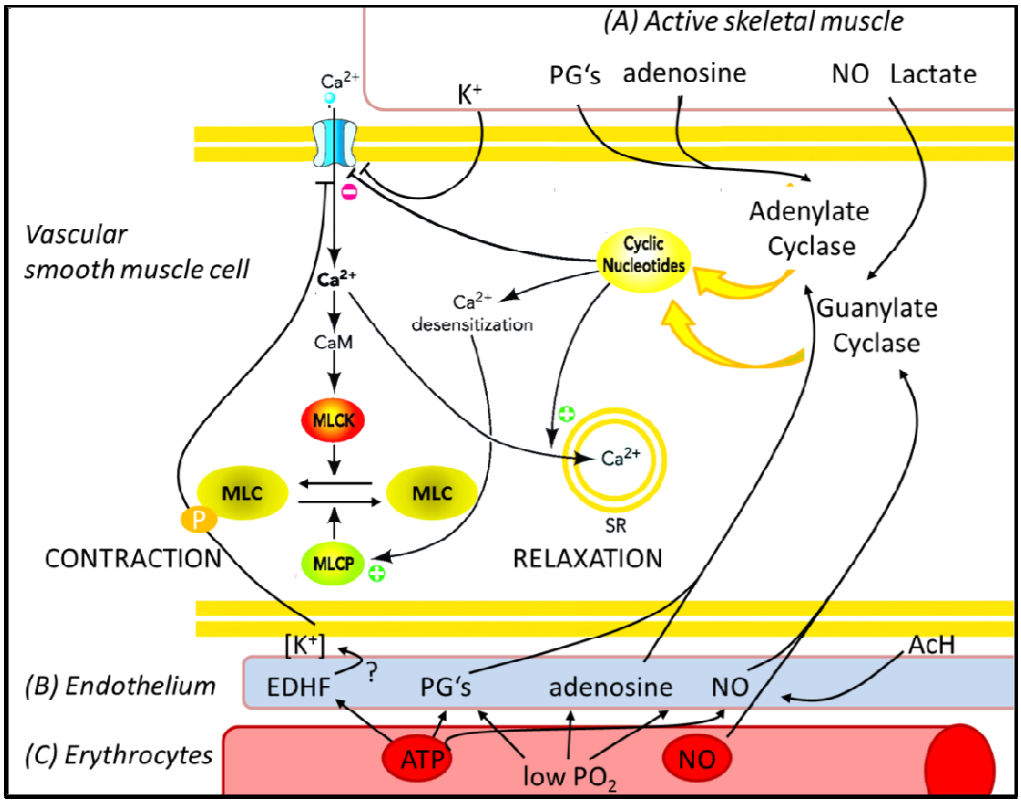

Figure 2. Simplified overview of metabolic factors and possible mechanisms involved in exercise-induced dilation of vascular smooth muscle. When Ca^{2+} concentrations are high, the Ca^{2+}-Calmoduline (CaM) complex activates myosin light chain kinase (MLCK), which in turn activates myosin light chain (MLC), leading to smooth muscle contraction. This mechanism is opposed by myosin light chain phosphatase (MLCP), which deactivates MLC and induces vasorelaxation. Mechanisms that induce vasodilation act via modulating Ca^{2+} concentrations and MLCP and MLCK activity. Smooth muscle relaxation can be induced via cyclic nucleotide production by adenylyl- or guanylyl cyclases. Substances like Prostaglandines (PG's) and adenosine activate adenylyl cyclase, whereas nitric oxide (NO) and lactate acitivate guanylyl cyclase. Vasoactive substances are derived from (A) active skeletal muscle and (B) endothelium or (C) erythrocytes. Endothelial cells (ECs) produce the endothelial-derived hyperpolarizing factor (EDHF), which induces vasodilation via smooth muscle membrane hyperpolarization. The '?' indicates that the mechanisms of EDHF-mediated vasodilation are to date mostly unsolved but are thought to involve increases in extracellular K^+. Reduced partial oxygen pressure (PO_2) in the blood can induce ECs to secrete vasoactive substances. Likewise, erythrocytes can produce vasoactive NO and ATP. Acetylcholine (AcH) is spilled over at neuromuscular endplates, which can induce NO production in ECs. See text above for more information. Modified from Clifford and Hellsten (2004) and Puetz et al. (2007) [24,25].

2.3.2.1 NO-mediated vasorelaxation

NO is a gaseous, easily diffusible radical [44], which has a half-life of only a few seconds [45]. NO is synthesized from the amino acid L-arginine involving molecular oxygen [46], Nicotinamide Adenine Dinucleotide Phosphate Hydrogen *(*NADPH) and the cofactors Tetrahydrobiopterin *(*BH4) and flavin mononucleotide cofactor (FMN) [47]. The formation of NO is catalyzed by nitric oxide synthases (NOS). To date, three types of NOS have been described: endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). In humans, nNOS is expressed in both fibre types of skeletal muscle, whereas eNOS is expressed in the endothelium [48]. Previous studies have shown that NO is not only involved in regulation of skeletal muscle blood flow during and in recovery from exercise [49–51] but also in adjustment of basal vascular tone in skeletal muscle [25]. Red blood cells are capable of performing enzymatic and non-enzymatic release of NO. In the former, NO disposal is accomplished through shear stress–induced activation of RBC-NOS and in the latter, NO release is induced in association with oxygen release and via reduction of circulating nitrite to NO by deoxygenated hemoglobin [52]. Vascular endothelial growth factor (VEGF) is an important stimulator of endothelial NO production and is capable to induce eNOS activation and gene expression [53]. It is commonly accepted that physical exercise elevates intravascular shear stress [54] and it has been shown that physical exercise activates RBC-NOS activity [55] and has a beneficial effect upon RBC deformability [56]. Furthermore, increased shear stress stimulates eNOS-mediated NO production in the endothelium [57,58]. Various studies have shown that shear stress affects eNOS mRNA and protein expression [27,59–61] and endothelium-dependent dilation was increased in soleus muscle feed arteries after an exercise intervention in rats [62].

2.4 Mechanisms of capillary growth

Vasculogenesis and angiogenesis are the two fundamental processes through which blood vessels are formed [63,64]. Vasculogenesis describes the *de novo* formation of blood vessels through differentiation of endothelial precursor cells (‘angioblasts’ in embryos and ‘endothelial progenitor cells’ in adults) into ECs, which assemble and form a primary vascular plexus. The term angiogenesis describes the outgrowth of new capillaries from pre-existing blood vessels and more generally, angiogenesis includes processes involved in growth and remodeling of a primitive network to form a complex vascular network [64,65], see Figure 3.

It is well established that both vasculo- and angiogenesis occur during embryonic development [64]. Formation of new blood vessels in adult organisms was for a long time thought to be restricted to angiogenesis, whereas recent findings describe the occurrence of postnatal vasculogenesis [66,67]. Blood vessel growth in adult organisms is found in pathological conditions such as wound healing, ischemia or cancer, but also in non-pathological conditions as a response to specific physiological situations like endocrine changes, e.g. in the female reproductive organs or in the placenta [68,69] or in muscle tissue as an adaptation to physical training [70].

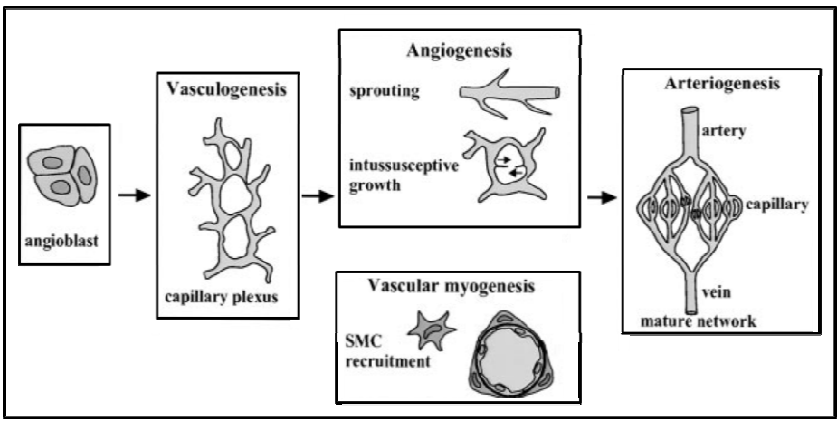

Figure 3. Formation of blood vessels. Angioblasts differentiate into endothelial cells, which assemble and form a primitive network, the capillary plexus, in a process called vasculogenesis. This primitive network matures through different modes of growth: via development and outgrowth of capillary sprouts (sprouting angiogenesis) or via longitudinal splitting of a pre-existing capillary (intussusceptive angiogenesis). By further maturation, including the recruitment of smooth muscle cells (SMC) and the construction of a basement membrane, a vascular network composed of arteries, capillaries and veins is established. Modified from Carmeliet & Collen, 2000 [71].

2.4.1 Angiogenesis

There are two processes through which angiogenesis may occur, namely via capillary sprouting or via intrussusception, i.e. longitudinal splitting of a pre-existing capillary [19]. While sprouting angiogenesis provides vascular supply to tissue areas previously devoid of blood vessels, intussusceptive growth allows the new-formation of capillaries in parts of tissue areas where capillaries already exist, see Figure 3.

Sprouting angiogenesis

In the current models of sprouting angiogenesis, capillary formation is described to begin with the activation of capillary ECs and proteolytic degradation of the basement membrane surrounding the capillary [72]. The resulting extension of the extracellular matrix (ECM)

enables sprout formation – i.e. ECs migrating into the interstitial matrix, where they proliferate towards an angiogenic stimulus such as VEGF [73]. Recent developments have shown that angiogenic sprouts are composed of so-called endothelial *tip*- and *stalk cells* [74]. Filopodia on *tip cells* express a large amount of VEGF receptors (VEGFR2) [75,76] and secrete proteolytic enzymes, so-called matrix metalloproteinases (MMP's), which digest the ECM [77]. Thus, these *tip cells* make the ECM accessible and via sensing of a VEGF gradient, the sprout is guided towards the angiogenic stimulus [78]. *Stalk cells* follow behind and proliferate, enabling the capillary sprout to elongate [74]. As the capillary sprout fuses with another sprout, a premature capillary lumen is formed and matures through further EC proliferation, by recruitment of pericytes and through the reconstruction of a basement membrane [79,80].

Splitting- or intussusceptive angiogenesis

Intussusceptive angiogenesis, also called splitting angiogenesis, describes a process in which interstitial tissue invades existing blood vessels from the luminal side, thus splitting a single capillary into two [81]. This type of angiogenesis is thought to be a very effective process, which requires less remodelling of the extracellular matrix compared to sprouting angiogenesis [82]. The mechanisms underlying intussusceptive angiogenesis are less well understood compared to sprouting angiogenesis.

2.4.2 Exercise-induced angiogenesis

Time course and extent of capillary growth varies according to the type of training performed. Generally, angiogenesis in skeletal muscle in response to exercise is thought to result from a complex interplay of various stimuli involving metabolic stimuli and the presence of a variety of growth factors [83] and mechanical forces acting on the microvasculature [84]. Yet, there is inconclusive evidence about which of the factors is initiating or maintaining capillary growth.

2.4.2.1 Mechanical stimulation of angiogenesis during exercise

As introduced in Figure 1, shear stress and wall tension are the main mechanical factors acting on the microvasculature, which have been implicated in inducing capillary growth [17]. Brown and colleagues [17] provided elegant rat models designed to decipher the angiogenic stimulus of intraluminal shear stress elevations versus increases in wall tension through elevated extraluminal forces. The essence of these studies was that elevated shear

stress induces blood vessel growth via capillary splitting and intussusceptive growth, including VEGF-associated EC proliferation but very little involvement of MMP's [85], whereas elevation of extraluminal forces induces capillary growth via proliferation and sprouting under involvement of both MMPs and VEGF [86]. In summary, MMP's were exclusively up-regulated by extravascular forces, whereas VEGF seems to be involved in both modes of capillary growth. Despite these findings, it is to date still unclear, which mode of capillary growth is induced by which type of physical exercise.

2.4.2.2 Metabolic stimulation of angiogenesis during exercise

Nitric oxide is activated by various stimuli derived during exercise, as overviewed in section 2.3.2.1 Endothelial NOS and its bioactive product NO are well-established pro-angiogenic agents [87,88], triggering EC proliferation and differentiation via cGMP-mediated activation of mitogen activated protein kinase (MAPK) and inducing basic fibroblast growth factor (FGF-2) expression [89].

As introduced in section 2.2, skeletal muscle activity leads to decreased PO_2. Hypoxia is a strong angiogenic stimulus which can induce the expression of the EC mitogen VEGF directly [90] or indirectly via the transcription factor hypoxia-inducible factor-1 alpha (HIF1α) [91]. HIF1α furthermore influences the transcription of VEGF receptor 1 (VEGFR1) [92], as well as iNOS [93]. Other examples for hypoxia-modulated angiogenic factors are transforming growth factor-beta (TGFß) [94], FGF-2 [95], placental growth factor (PlGF)[96], angiopoietin (Ang) -1 and -2 [90], and endostatin [97].

The nucleotide adenosine is found in all cells and is formed by stepwise dephosphorylation of adenosine triphosphate (ATP). Exercise-induced hypoxia favors the production of adenosine, which acts as both a vasoactive substance and as a pro-angiogenic factor, being capable of inducing EC proliferation and –migration under hypoxic conditions *in vitro* [98,99] and blood vessel growth *in viv*o [100], possibly via VEGF [101].

2.4.3 Angiogenic factors

2.4.3.1 Vascular endothelial growth factor (VEGF)

The VEGF gene family consists of six members: VEGF-A, VEGF -B, -C -D, and -E and placental growth factor (PIGF) [102]. VEGF-A is a potent angiogenic factor and EC mitogen as well as a major regulator of EC function, being capable to stimulate EC differentiation, proliferation, migration, and survival, vascular permeability and NO production [103–106]. VEGF-A was

measured in serum after exercise in the present thesis and therefore, the following paragraph will focus on this family member.

The VEGF-A gene is organized in eight exons and seven introns [107] and is expressed in various cell types, e.g. ECs, vascular smooth muscle cells, macrophages, fibroblasts as well as cardiac and skeletal muscle cells [90]. Via alternative splicing, five different VEGF-A protein isoforms are generated, containing 121 to 206 amino acids (VEGF-$A_{121\text{-}206}$) [107]. Heparan-sulfate proteoglycanes residing within the extracellular matrix are thought to function as an extracellular storage modality for VEGF-A isoforms possessing a heparin binding motif [108]. The different VEGF-A isoforms have differential affinities to heparin sulfate. In contrast, the shortest isoform VEGF-A_{121} does not bind to heparin and is therefore freely diffusible while the largest isoforms VEGF-A_{189} and VEGF-A_{206} are tightly bound to heparin-containing proteoglycanes. VEGF-A_{165} is the predominant isoform and has intermediate properties, which means that upon secretion, a significant fraction remains associated to the cell membrane and extracellular matrix [108]. This enables the establishment of extracellular VEGF-A pools of which bioavailability is triggered upon ECM degradation [109]. Furthermore, VEGF-A can stimulate the production and secretion of matrix degrading enzymes such as the matrix metalloproteinases in endothelial and smooth muscle cells and thus facilitating EC migration and tube formation [110]. VEGF-A is EC-specific where its tyrosine kinase receptors VEGFR-1 and VEGFR-2 are expressed [111] and VEGF binding promotes EC survival via activation of phosphatidylinositol-3 kinase (PI3K) and Akt [112]. EC proliferation is induced mainly via Phospholipase C (PLC) - MAPK pathway. VEGF is furthermore known to induce NO production via activation of eNOS through phosphorylation by AKT or via calcium influx induced by PLC [113].

Previous research reveals that VEGF-A is activated upon elevated shear stress perturbation [114], muscle stretch [115] and hypoxia [116] and furthermore, VEGF-A has been shown to be essential for exercise-induced angiogenesis in skeletal muscle [117,118]. Exercise leads to increases of VEGF-A protein concentrations [119] and mRNA [119–121] both within skeletal muscle fibres and also in interstitial cells between the muscle fibres [120,121]. VEGF-A is released by active skeletal muscle [122] and serum levels of VEGF-A were shown to be unaffected [123], decreased [119,124] or elevated [125,126] after endurance-type exercise. So far, serum levels of VEGF-A induced by resistance exercise have not been tested, and this

task was one aim of the present thesis. In the following, VEGF-A will be referred to as simply "VEGF".

2.4.3.2 Matrix Metallo Proteinases (MMP)

The family of zinc- and calcium-dependent enzymes known as MMPs are extracellular proteinases that play important physiological roles in extracellular matrix remodeling during development, tissue remodeling, angiogenesis and during pathological conditions such as wound healing, inflammatory disease and tissue invasion by tumors [127]. Major characteristics of MMPs are that (i) they are synthesized as zymogenes, which are activated upon proteolytic removal of an amino-terminal propeptide [128]; (ii) the presence of the co-factor zinc in the active site is essential for their activity; (iii) MMP activity is inhibited by specific inhibitors of metalloproteinases and (iv) MMPs collectively degrade all major components of the ECM [129]. Several subclasses of MMPs have been described including collagenases, gelatinases, membrane-type metalloproteinases and stromelysins [129]. The present thesis deals with the effect of exercise on serum levels of gelatinases. Therefore, the following passage will focus on this subclass.

The gelatinase subgroup has two members, namely MMP-2 /gelatinase A [130] and MMP-9 / gelatinase B [131]. MMP-2 and -9 are expressed in a large variety of tissues, MMP-2 being produced by chondrocytes, fibroblasts, keratinocytes and monocytes [130] and MMP-9 by e.g. keratinocytes, monocytes and leukocytes [132]. Of note, both gelatinases are produced in ECs and are thought to play a vital role in initiating angiogenesis [133,134]. The importance of MMPs in the angiogenic process has been underlined by several studies showing that inhibition of MMP activity disrupted angiogenesis [135–138]. The proteases MMP-2 and -9 seem to play a crucial role in the formation of new capillaries in skeletal muscle [139,140] and their plasma concentrations were significantly elevated in serum after endurance exercise [133,141–144]. MMPs have also been implicated in the release of growth factors such as VEGF [145], transforming growth factor beta (TGFß) or FGF [146]. Previous *in vitro* research reveals that MMP-9 is more prone to release VEGF compared to MMP-2 [145]. Moreover, MMP-2 has shown to increase the bioavailability of insulin-like growth factor (IGF) through proteolysis of the IGF binding protein and might therefore be involved in anabolic stimulation of skeletal muscle [147,148].

Upon ECM cleavage, bioactive degradation products are generated which have signaling properties [149,150]. One of these factors is the angiogenic factor endostatin which will be introduced in the following paragraph.

2.4.3.3 Endostatin

Endostatin corresponds to the C-terminal fragment of the non-collagenous (NC1) domain of type XVIII collagen [151] and is proteolytically released by proteases like cathepsins, elastase, MMP-2 and -9 [149,152]. Endostatin has a strong affinity to heparan sulfate proteoglycans, which determines its localization at capillary basement membranes [153]. Endostatin was first identified in murine hemangioendothelioma cells as an 'endogenous inhibitor of angiogenesis' and it has been shown that endostatin inhibits primary and metastatic tumor growth in animal models [154–156]. To date, the role of endostatin in the angiogenic process is not clear due to its complex signaling functions and because both pro-angiogenic and anti-angiogenic characteristics have been described [157].

The anti-angiogenic effect of endostatin may be exerted via its interaction with integrin α5β1, resulting in inhibition of matrix adhesion and signaling [158], involving down regulation of MAPK and focal adhesion kinase (FAK) pathways [188]. Moreover, endostatin inhibits EC proliferation and –migration via competitive inhibitory binding to VEGFR2 [159] and suppression of Wnt signaling [160]. Endostatin has also been shown to inhibit EC proliferation via inducing cell cycle arrest and down regulation of cyclin D1 [189]. In addition, endostatin action has been attributed to induce EC apoptosis, possibly via reducing anti-apoptotic proteins [159].

Studies performed by Schmidt and colleagues (2004) reveal that endostatin can elicit pro-migratory and pro-proliferative effects on ECs and that the pro- or anti-angiogenic effect of endostatin is determined by its concentration and the proliferation status of ECs [157]. Isolated ECs and endothelial progenitor cells from hemangiomas showed increased adhesion, proliferation and migratory activity in response to endostatin treatment [161]. Moreover, endostatin was shown to increase NO release from ECs *in vitro* a[162] and local levels of endostatin may therefore be crucial for regulation of peripheral vascular tone. These studies collectively reveal that endostatin might function as an angiogenic modulator rather than an anti-angiogenic agent [157].

Collagen turnover has been reported to be increased by physical exercise [163] and previous studies reveal that endostatin seems to play a role in exercise-induced adaptations, as serum

concentrations were acutely elevated after endurance exercise [124,144,164]. Moreover, long-term endurance exercise was shown to decrease basal levels of circulating endostatin in healthy [144] and obese [165] men.

2.4.3.4 Interplay of the angiogenic factors that were measured in the present thesis

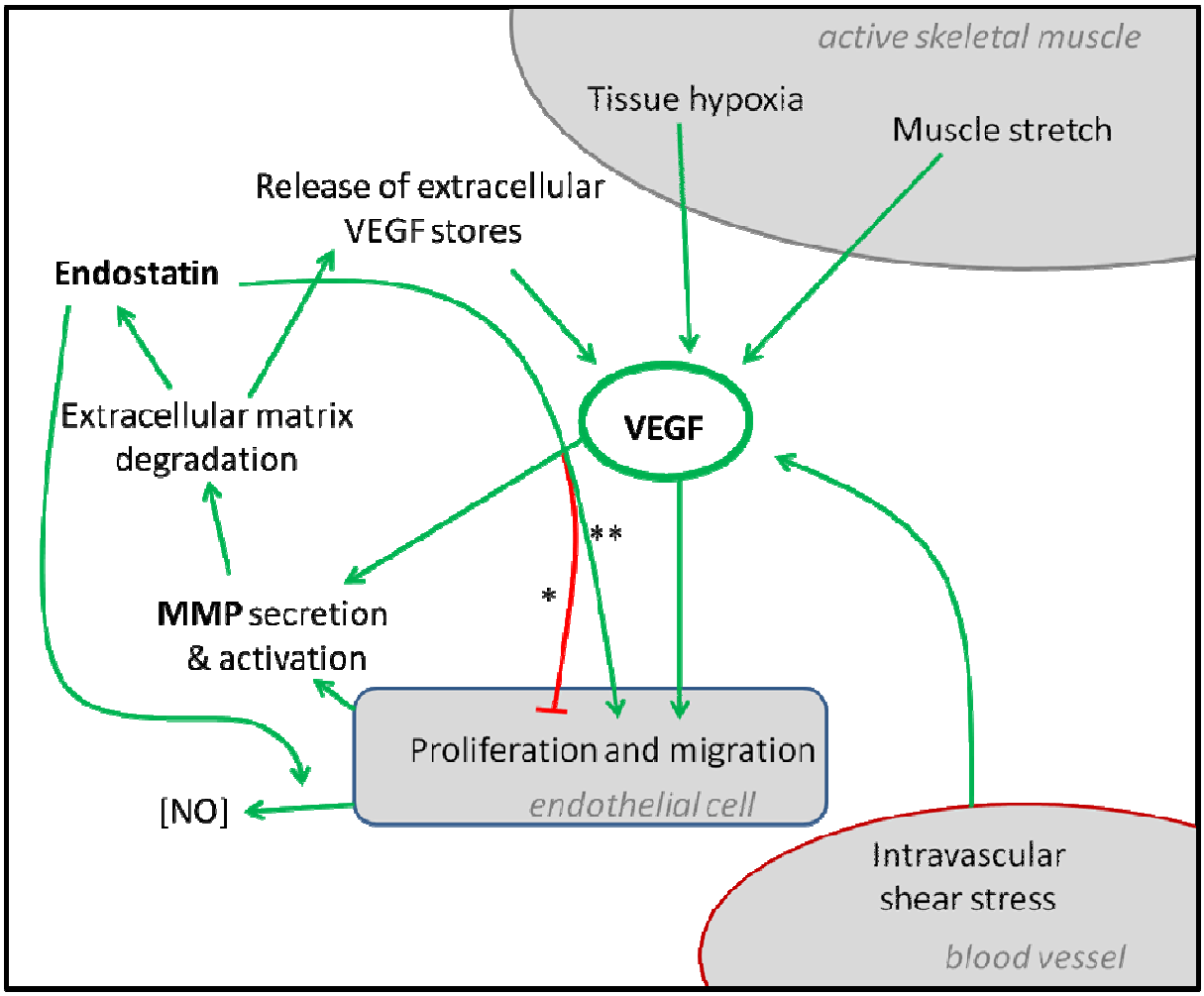

Figure 4. Simplified overview of the interplay of the angiogenic factors that were measured in the present thesis. Vascular endothelial growth factor (VEGF) is induced by stimuli that arise during physical exercise such as tissue hypoxia, muscle stretch and intravascular shear stress. VEGF binding to its receptor VEGFR2 induces EC proliferation and migration. VEGF furthermore induces secretion and activation of matrix metalloproteinases (MMP), which degrade the extracellular matrix and create a lumen into which endothelial cells can migrate to form a novel capillary sprout. Upon ECM degradation, extracellular VEGF stores are released and ECM degradation products such as endostatin are generated. Endostatin functions as an angiogenic modulator which can both inhibit (*) and induce (**) and EC proliferation and migration. The latter is achieved via competitive inhibitory binding to VEGFR2. Endostatin has furthermore been reported to increase NO release from ECs *in vitro* and may be involved in regulation of peripheral vascular tone.

3 Purpose of the Thesis

The training study *Molecular and functional **E**ffects of **V**ibration **E**xercise* ('EVE') was conducted within the scope of the present thesis. The study was designed to evaluate effects of resistance exercise with and without superimposed whole-body vibrations (WBV) on the microvasculature of skeletal muscle. The underlying idea was derived from the field of aerospace medicine where vibration training in combination with resistance exercise has proven to prevent disuse-induced adaptations in skeletal muscle, such as myofibre atrophy [166,167] and capillary loss [168–170]. Hence, WBV is currently being considered as a potential training modality for human space flight [171]. WBV training is nowadays commonly applied in various fields like sports, preventive medicine and rehabilitation [171,172] and has been described to improve neuromuscular performance [173–175]. Whole-body vibration exercise has also been shown to moderately increase metabolic activity and ATP consumption [176–179] and to elicit muscle deoxygenation [180,181]. Previous studies reveal that the mechanical stimulus of WBV increases blood viscosity [182] and may have beneficial effects upon peripheral perfusion as blood flow velocity was increased in skeletal muscle during and immediately after termination of WBV [183,184] . Increased viscosity and blood flow may all result in an elevated shear stress in the microvasculature of skeletal muscle [172,182], as overviewed in section 2.2..

Based on the finding that shear stress and hypoxia are able to induce angiogenesis [19,185,186] and inspired by a previous study showing increased circulating VEGF concentrations upon WBV exposure during cycling exercise [164], we hypothesized that the superposition of a WBV stimulus to resistance exercise would add a pro-angiogenic stimulus to the training. To test this hypothesis, 26 healthy male subjects were subjected to a 6-week training intervention, in which 13 subjects performed resistance exercise (RE) and 13 subjects performed resistive vibration exercise (RVE). High resistance training has in previous studies been shown to decrease capillary density probably as a result of fibre hypertrophy with insufficient angiogenesis [187]. Hence, we aimed to find a novel training mode that concurrently increases muscle strength and induces capillary growth to optimize the flux of oxygen and nutrients to the muscle and thereby improve muscle performance in terms of power output and endurance capacity.

The present thesis is organized as follows:

- Paper 1 outlines the study design, feasibility and demands of the study as well as cardiovascular adaptations to the two training regimes.
- Paper 2 highlights changes in circulating concentrations of the angiogenic factors MMP-2, MMP-9, VEGF and Endostatin and their effect upon EC proliferation. We hypothesized that superposition of WBV to resistance exercise would enhance circulating concentrations of pro-angiogenic factors acutely after training, which would induce more pronounced EC proliferation *in vitro*.
- The purpose of Manuscript 1 was to evaluate acute and long-term effects of the training interventions upon skeletal muscle microcirculation and capillary growth in calf muscles. We hypothesized that (i) superposition of WBVs to RE would induce muscle deoxygenation and increase exercise-induced hyperaemia compared to training without WBV and (ii) that this effect would lead to more pronounced long-term adaptations in the RVE group, leading to increased capillarity and improved perfusion of skeletal muscle.

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4 Human studies

4.1 Paper 1: 'Randomized controlled study on Resistive Vibration Exercise (EVE Study): Protocol, Implementation and Feasibility'

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Abstract

Objectives: A training intervention comparing resistance exercise with or without whole-body vibration (WBV) was conducted to compare acute and chronic effects on functional and molecular parameters.

Methods: A six-week training intervention was performed including 26 healthy males (26 years, SD=4). Two groups were analyzed in a parallel design performing either resistive exercise (RE, n=13) or resistive vibration exercise (RVE, n=13) training with weekly increasing vibration frequencies (20-40Hz). Resting and exercising blood pressure and heart rate were measured before and after the 6-week intervention.

Results: Both training interventions decreased resting systolic blood pressure (P = 0.003). Resting diastolic blood pressure was significantly decreased only in the RVE group (P = 0.01). Exercising diastolic blood pressure was significantly decreased during the final training (P<0.001) with no additional effect of superimposed vibrations. Resistance exercise with superimposed vibrations evoked back pain to a higher degree than resistance exercise alone when training at frequencies above 30Hz (P<0.01).

Conclusions: These data suggest positive effects of resistance exercise upon cardiovascular health and vascular responsiveness and a further beneficial effect of superimposed vibrations in decreasing resting diastolic blood pressure. Finally, development of back pain may be fostered by superimposed vibrations to high training loads, particularly at higher frequencies.

INTRODUCTION

Regular performance of aerobic exercise is commonly known to have beneficial effects upon cardiovascular health such as decreases in heart rate and ambulatory blood pressure [1,2]. However, studies on cardiovascular adaptations to resistance exercise remain inconclusive. In the early 1980's, resistance exercise was believed to cause hypertension [3]. However, other studies showed that resting blood pressure was decreased by a resistance training intervention [4,5] whereas other studies showed no effect upon resting blood pressure in normotensive individuals [6–8]. The divergence in the reported effects indicates the need for further investigations in the field of cardiovascular adaptations to resistance exercise. Here we report acute and long-term responses of blood pressure and heart rate to a resistance training intervention performed with and without superimposed vibrations. Whole-body vibration (WBV) training has become increasingly popular during the past two decades and is nowadays applied in various fields like sport, rehabilitation and in clinical settings. Previous studies have made a great effort to describe physiological effects of whole-body vibration and have been reviewed elsewhere [9,10]. Unfortunately, many of the reported vibration-induced effects vary from study to study, which may derive from discrepancies in the applied training protocols, subject heterogeneity and divergence in the duration of the interventions. Furthermore, training supervision and diet control were neglected in many of the studies, and there was likewise no uniformity in the control conditions: studies either lacked a control group or compared their results to a passive control group; only few studies applied an exercise control condition [10]. Also, there is a lack of consistency in the way of reporting the results, as highlighted in the recommendations of the international society of musculoskeletal and neuronal interactions [11]. Many of the potential benefits of whole-body vibration may thus not have been clearly demonstrated. To the best of our knowledge, no study has yet compared acute effects of a specific exercise to its long-term adaptations. However, and considering that exercise is usually conducted regularly and over a longer period of time, it is pertinent to ask whether long-term training alters acute responses and if superimposed vibrations promote a beneficial training effect. Here we present the design, feasibility and demands of a conducted study that allows investigation of the adaptation of acute responses during exercise to a long-term training intervention. Acute functional parameters (cardiovascular responses, neuromuscular activation, oxygen consumption, muscle perfusion and oxygenation) are complemented with investigations of acute

responses on circulating factors in serum as well as acute and long-term responses within muscle tissue. The various measurements within a single training study using an exercising control group will hopefully provide a broader insight into the effects of the vibration stimulus per se. The present article focuses on acute and long-term cardiovascular responses as well as feasibility and demands of the training.

MATERIAL AND METHODS

Study design:

The EVE study ("Molecular and functional Effects of resistive Vibration Exercise") was conducted in a two-group parallel design and was carried out in compliance with the Declaration of Helsinki following approval by the Ethics Committee of the Northern Rhine medical association (Ärztekammer Nordrhein) in Düsseldorf (application no. 2010-174). After providing a written informed consent, 28 healthy male subjects were included into the study and stratified according to their vertical jumping height into two matched groups with comparable neuromuscular fitness, using the maximum vertical jump height as an indicator [12]. A coin was then tossed to determine which group would perform either resistive vibration exercise (RVE) or resistive exercise (RE) only. The study was conducted in two campaigns due to feasibility reasons: the first campaign with 12 subjects took place between October 2010 and March 2011, the second campaign with 16 subjects took place between May and October 2011.

Participants and group design

Healthy, male subjects were targeted who were recreationally physically active (exercised 2-3 times per week). Any competitive sports, participation in strength training during the past six months, smoking, diabetes as well as any current medication were considered as exclusion criteria. Subject recruitment involved a telephone questionnaire checking for general suitability (224 applicants), a medical screening comprising a short medical history, blood analysis (involving a complete blood count and investigation of clinical parameters - creatinin, urea, protein, albumin, SGOT, SGPT, γGT, Lipase, alk. phosphatase, electrolytes, glucose, C-reactive protein and haematological parameters: PTT, aPTT, Quick, INR), as well as a urine test checking for glucose, protein and urobilinogen. Finally, a stress electrocardiogram on a cycling ergometer and a training familiarisation were performed. The medical screening involved 60 applicants out of which 28 were included in the study. The

subject's anthropometric data at baseline are given in Table 1, and no statistically significant group difference was found ($P > 0.08$).

	RE group (n = 13)	**RVE group (n = 13)**	***P* - value**
Age [yrs]	23.4 (± 1.4)	24.3 (± 3.3)	0.52
Body mass [kg]	75.0 (± 4.7)	74.7 (± 6.9)	0.08
Height [m]	1.79 (± 0.05)	1.8 (± 0.1)	0.31
BMI	23.4 (± 1.4)	23.5 (± 2.1)	0.11
CMJ height [cm]	42.2 (± 4.6)	41.7 (± 2.2)	0.97
Maximal performance on cycle ergometer test [W / kg body weight]	3.3 (± 0.3)	3.3 (± 0.4)	1.00

Table 1. Anthropometric data of EVE subjects at baseline. BMI: Body Mass Index., CMJ: Counter movement jump. There was no difference between the two groups.

Training design:

The present study was designed to compare acute and long-term effects of two training interventions: Resistive Exercise (RE) and Resistive Vibration Exercise (RVE). Subjects trained for six weeks, 2-3 times per week with additional weights. In order to align the squatting movement, the weights were put on a guided barbell (PTS Dual action Smith, Hoist, U.S.A). A vibration platform (Galileo® Fitness, Novotech, Germany) was placed underneath, as illustrated in Figure1A. The subjects in the RVE group performed the resistive exercise training protocol with simultaneous side-alternating whole-body vibrations, whereas subjects of the RE group trained with the same setting, without superimposed vibrations. We aimed to test physiological responses at 40Hz side-alternating vibration, which has not been tested before. Preliminary testing yielded that this is challenging for people not acquainted with whole-body vibration. We therefore decided to initially set the vibration frequency to 20 Hz and to increase the vibration frequency throughout the study to eventually arrive at 40Hz.

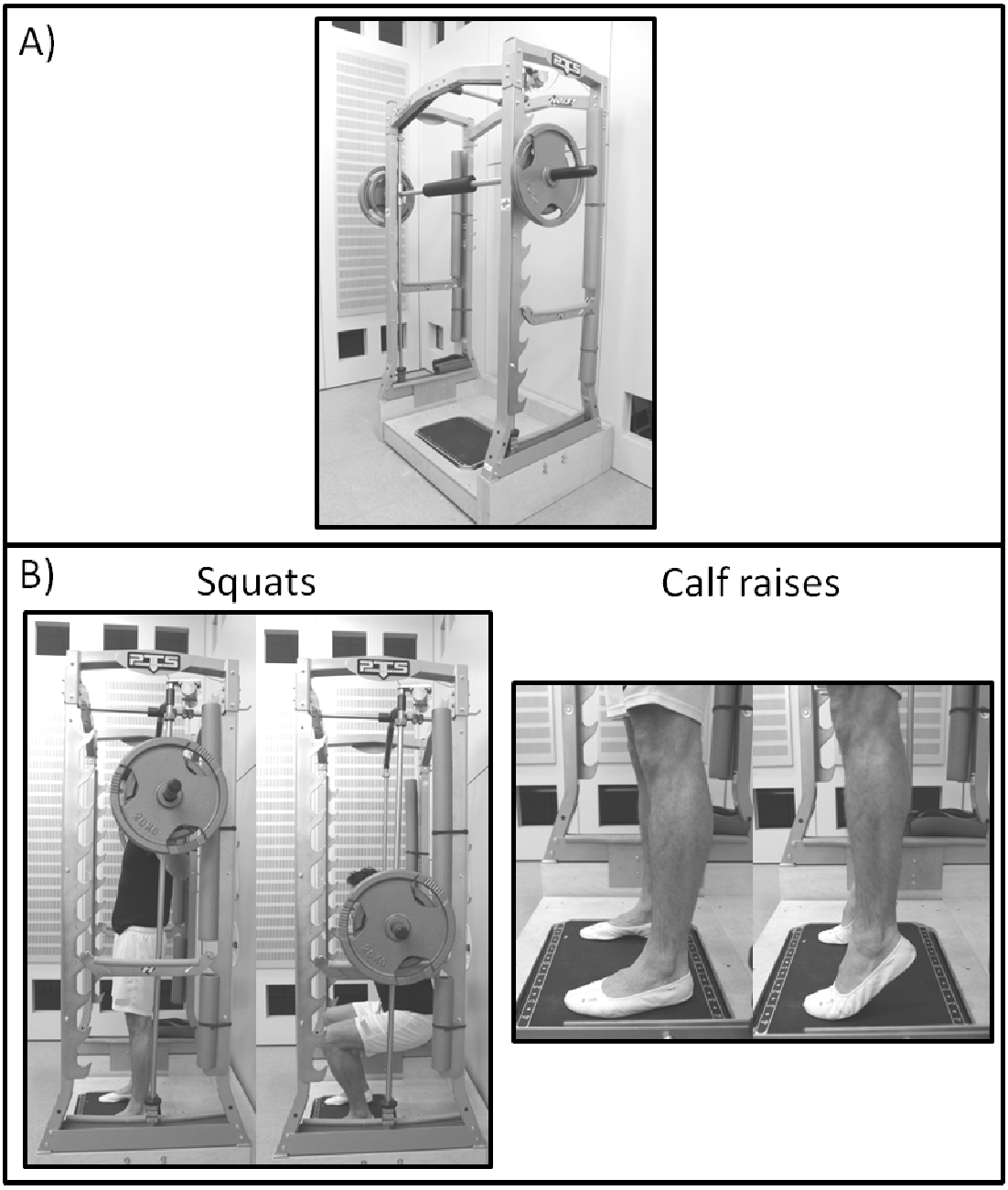

Figure 1. (A) Illustration of the training device. A guided barbell with a vibration plate placed underneath, embedded into a custom-built frame. **(B)** Illustration of the exercise movements. Squats (left) and calf raises (right).

Estimation of training load

The individual training load was set at 80% of the subjects One-Repetition Maximum (1-RM), which was estimated in the familiarisation session four weeks prior to the first training, applying the method of Baechle and Earle [13] and performing squats in a non-vibrating condition.

Briefly, the guided barbell was initially loaded with weights corresponding to the subject's body weight plus 20kg and subjects were asked to perform as many squats as possible. The

corresponding % of the 1-RM was evaluated according to Baechle and Earle [13]. An example is illustrated in Figure 2: if the barbell was loaded with 90kg and the subject's maximum number of repetitions was 5, which corresponds to 87% of the 1-RM, the training load was adjusted to 85kg.

% of the 1-RM	Repetitions
100	1
95	2
93	3
90	4
87	5
85	6
83	7
80	8
77	9
75	10

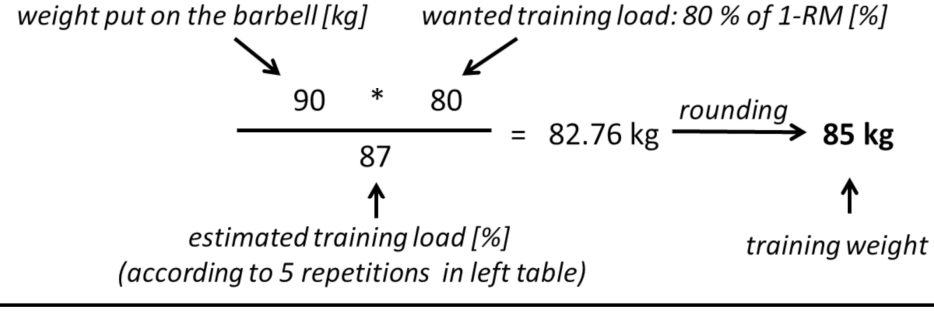

Figure 2. Determination of training load. Left: calculation of the performed % of the One-Repetition. Maximum (1-RM) according to the number of concluded repetitions (adapted from Baechle and Earle). Right: example for estimation of training load at 80 % the 1-RM.

Training protocol

The training was supervised by a graduated exercise scientist throughout the study and two spotters were standing left and right of the guided barbell providing subject security. A metronome guided the training rhythm to provide standardisation of the movement. Squats were performed dynamically with 2 sec. eccentric and 2 sec. concentric phase; calf raises were performed with 1 sec. eccentric and 1 sec. concentric phase (Figure 1B). Each training session included a warm-up with the unloaded barbell (15kg), which consisted of two sets; each set with 10 squats and 15 heel raises. The actual training was performed in three sets: the first two sets comprised 8 squats and 12 calf raises; in the third set, as many squats and calf raises as possible were performed (Figure 3A). Immediately after completion of the last set of squats, each subject's perceived exertion was rated via the Borg RPE Scale [14]. Altogether, the subjects concluded 16 training sessions in a period of 6 weeks (week 1-2: two sessions per week; week 3-6: three sessions per week). Both training regimens differed only in the vibration component.

Increase of training load and vibration frequency during the 6-week intervention

The number of squats in the third set was used to readjust the training weight to 80% of the 1-RM for the following training. When the number of squats in the third set was equal to 8, the training weight remained unchanged for the subsequent training. When the subjects performed more or less than 8 repetitions, the training weight was recalculated, i.e.

increased or decreased for the next training. However, the top limit for weight increases was set at 10kg in order to guarantee steady weight increments. The RVE group started the training with 20Hz vibration with weekly increments by 5Hz; during the last two weeks, vibration frequency was set at 40Hz. A schematic overview of the incremental study design is displayed in Figure 3B.

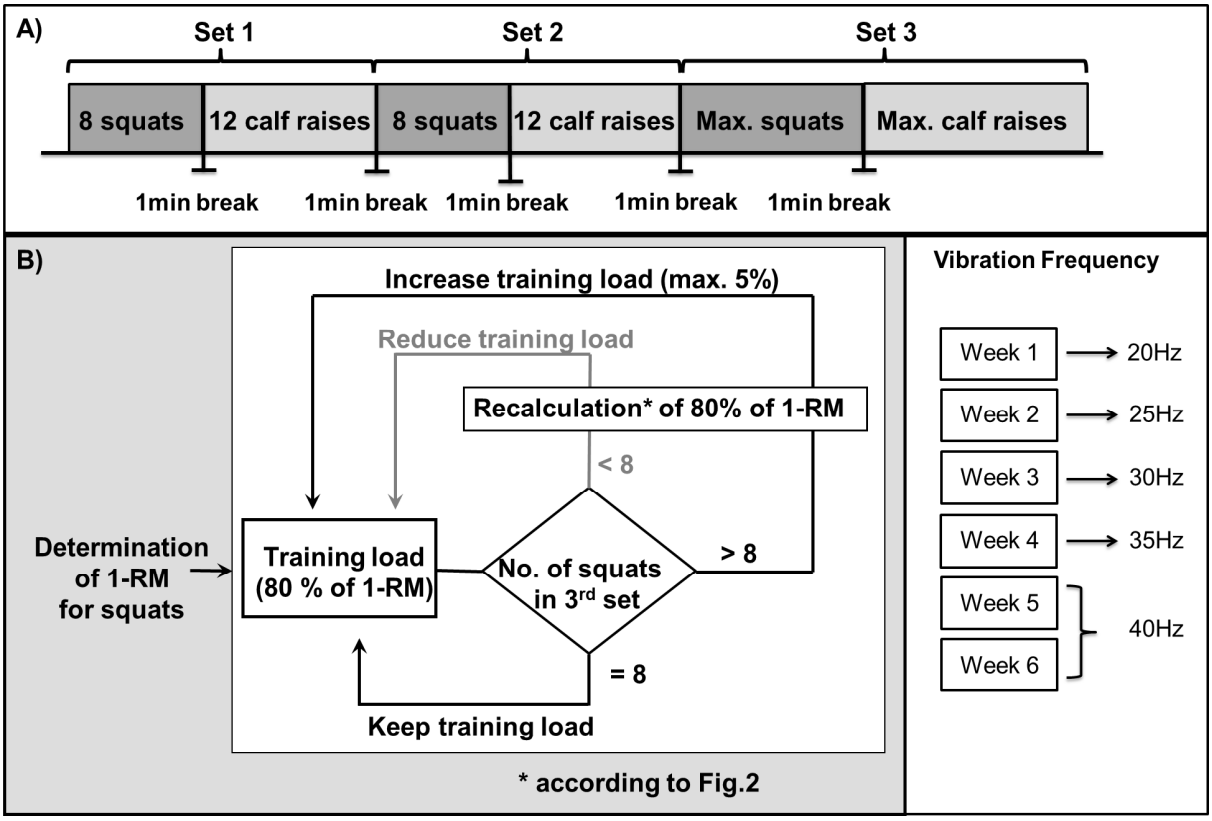

Figure 3. (A) Training design. After a warm-up, subjects performed three sets of squats and calf raises. The first two sets included 8 squats and 12 calf raises, in the third set, a maximum number of squats and calf raises was performed. **(B)** Increase of of training intensity over the 6-week training intervention. Left: increase of training load for both intervention groups. Right: increase of vibration frequency in the resistive vibration exercise (RVE) group. 1-RM: One-Repetition Maximum.

Diet

During the initial and final training sessions, subjects ate a standardised breakfast two hours before training (two wheat bread rolls with butter and jam). During the long-term training intervention, subjects were asked to abstain from food two hours before every training session and to drink a protein energy drink (Fresubin® protein energy drink, Fresenius Kabi, Germany) one hour prior to training.

Measurements

The present study was designed to characterize the acute and long-term effects of resistive exercise and superimposed vibrations on both functional and molecular levels. An overview of the measurements with the corresponding time points is depicted in Figure 4.

Determination of daily physical activity

The Freiburg Questionnaire [15] was applied to assess the subject's daily physical activities. Subjects filled the questionnaire one week prior to and three days after the 6-week training intervention.

Blood pressure and heart rate at rest and during exercise

Resting heart rate and blood pressure were recorded after 20 minutes in horizontal position with an automated sphygmomanometer (Medicus pc, Boso, Germany). Exercise blood pressure was measured during each break between the sets and immediately after training termination by a medical doctor using a manual sphygmomanometer. Heart rate was measured manually by an exercise scientist.

Rating of perceived exertion (RPE)

The Borg RPE scale[14] was used for the assessment of the perceived exertion of the training. Within 20sec after the last set of squats, subjects provided their individual RPE.

Study Week	-4	-3	-2	-1	1		2		3			4			5				6			+3d	+4d	+90d
Measurement / Training no.	BDC			Initial Training	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Final Training		Follow-up measurements		
Vibration frequency				20 Hz	20 Hz		25 Hz		30 Hz			35 Hz			40 Hz									
Diet (s=standardised, f=fastened)		f	f	s					f						f				f	s		f	f	
Protein Drink (1h pre training)					x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x				
Freiburg Questionnaire			x																			x		
Rating of Perceived Exertion				x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x				
Estimation of training load	x																							
Familiarisation Session	x																							
Body Weight Determination			x																			x		
Jump tests	x																					x		x
Doppler Ultrasound		x							x						x				x			x		
MRS		x																				x		x
MVC		x																				x		x
MRI			x																				x	x
Gait Analysis			x																x					
Blood Pressure, Heart Rate				x																x				
Lactate Test	x			x					x						x					x		x		x
Spirometry during Training				x																x				
EMG during Training				x																x				
NIRS during Training				x																x				
Serum Collection				x																x				
Muscle Biopsy			x	x																			x	

Figure 4. Overview of the EVE-Study design. BDC (Baseline Data Collection) was performed during 4 weeks prior to the initial training; follow- up measurements were performed 3, 4 and 90 days (d) after the final training. MRS: Magnetic Resonance Resonance Spectroscopy, MVC: Maximal Voluntary Contraction, MRI: Magnetic Resonance Imaging, EMG: Electromyography, NIRS: Near-Infrared Spectroscopy.

Statistical analyses

Statistical analyses were performed using STATISTICA 10 for Windows (Statsoft, Tulsa, Oklahoma, USA, 1984-2010). For estimation of differences in training load increments, rating of perceived exertion, blood pressure and heart rate, a repeated measures ANOVA was applied with time (initial vs. final) and intervention (resistive exercise vs. resistive vibration exercise) as factors; Tukey's test was used for post-hoc testing. For estimation of daily physical activity (Freiburg Questionnaire), a paired, two-sided Student's t-test was performed to compare physical activity before and after of the 6-week training intervention; an unpaired, two-sided t-test was performed to test differences between the two intervention groups. For estimation of vibration-induced back pain, a chi-square analysis was performed. Values are given as means ± standard deviation, statistical significance was set at $P < 0.05$.

RESULTS

Freiburg Questionnaire of physical activity

Daily physical activities like walking, biking, stair climbing, activity at work, sleeping and weekly sportive physical activity did not differ before and after the 6-week training intervention (*P* – values between 0.12 and 0.96) and did not differ between the two intervention groups (*P* – values between 0.32 and 0.75).

Important events during the study

When training at frequencies above 30Hz, eight of the RVE subjects complained about back pain. In one of the subjects, back pain was the cause for dropping out of the study. The sudden onset of back pain in the drop-out subject was caused by an incident during training. The impression of the personal trainer and his assistants present during that exercise session was that the incident resulted from training with poor body balance, which led to bending of the back. An independent orthopaedic surgeon diagnosed a facet joint syndrome L1-2, which did not implicate sensory or motor deficits. The back pain lasted for seven days after the incident and was ranked by the subject to an intensity of 8 using a scale ranging from 0 to 10, where 0 indicated “no pain” and 10 indicated “severe, unbearable pain”. The subject had demonstrated questionable commitment before that event, which reinforced the decision was made to exclude him from the further participation.

Back pain reported by the other seven subjects that completed the study successfully was assessed via a questionnaire. All seven subjects reported low back pain without radiculopathy. One subject complained about pain during training, whereas the majority (6 out of 7 subjects) perceived back pain after training termination. The duration of the pain varied: two subjects reported acute pain until 1-2 hours after training, and four subjects reported pain until 2-3 days after training. The pain intensity estimated by the subjects ranged from 3 to 7 and was on average 4.4 (SD=1.4), using a 0-10 scale (as described above). None of the subjects had to take analgetics to relieve the pain. There were only two cases of back pain in the RE group: one subject complained about local neck pain at the site of weight application, the other subject complained about “light” muscle tenderness in the lumbar spine. Statistical analyses revealed that resistive vibration exercise at frequencies of 30 Hz and above caused back pain in a higher number of cases than resistive exercise alone (Chi-value < 0.01); details are listed in Table 2.

Furthermore, four subjects in the RVE group complained about a training-induced headache with an onset after the second training set, out of which one subject dropped out after four weeks of training because of a headache that was reproducibly generated by the combination of vibration, application of the bar bell and calf raises. A post-hoc medical check revealed the absence of the physiological lordosis of the cervical spine as a likely explanation for this reaction.

Training week	Vibration Frequency	**Back Pain**		**Headache**	
		RVE	RE	RVE	RE
1	20 Hz	-	-	-	-
2	25 Hz	-	-	-	-
3	30 Hz	3	1	1	-
4	35 Hz	1	1	2	-
5, 6	40 Hz	4	-		-
	Sum	8**	2	3	0

Table 2. Important events during the study. Numbers of subjects are indicated perceiving headache or back pain in the respective training week. RE: resistive exercise group; RVE: resistive vibration exercise group. **Higher compared to RE group (chi-value<0.01).

Conduct of exercise: missed training sessions

In the RVE group, four subjects completed all 16 training sessions and nine subjects missed a single training session. In the RE group, ten subjects completed all 16 training sessions and three subjects missed a single training session.

Increase of training load

The training loads were comparable between the two groups at the initial training (RVE: 81.5 ± 7.7 kg, RE: 75.2 ± 6.5 kg; $P = 1.0$) and increased over time in both groups ($P < 0.001$). Compared to the initial training, the increase in training load over the six-week training intervention was significantly higher in the RE group and accounted for 59.8 ± 17.3 %, compared to 46.9 ± 19.0 % in the RVE group (time * intervention: $P < 0.001$). As the weight increase was more pronounced in the RE group, post-hoc analyses reveal that RE subjects trained with significantly higher training loads compared to the RVE group in trainings 13 to 16 ($P < 0.01$). During the final training, the RE group trained with 130.2 ± 18.5 kg and the RVE group trained with 110.2 ± 15.8 kg ($P = 0.003$), see Figure 5.

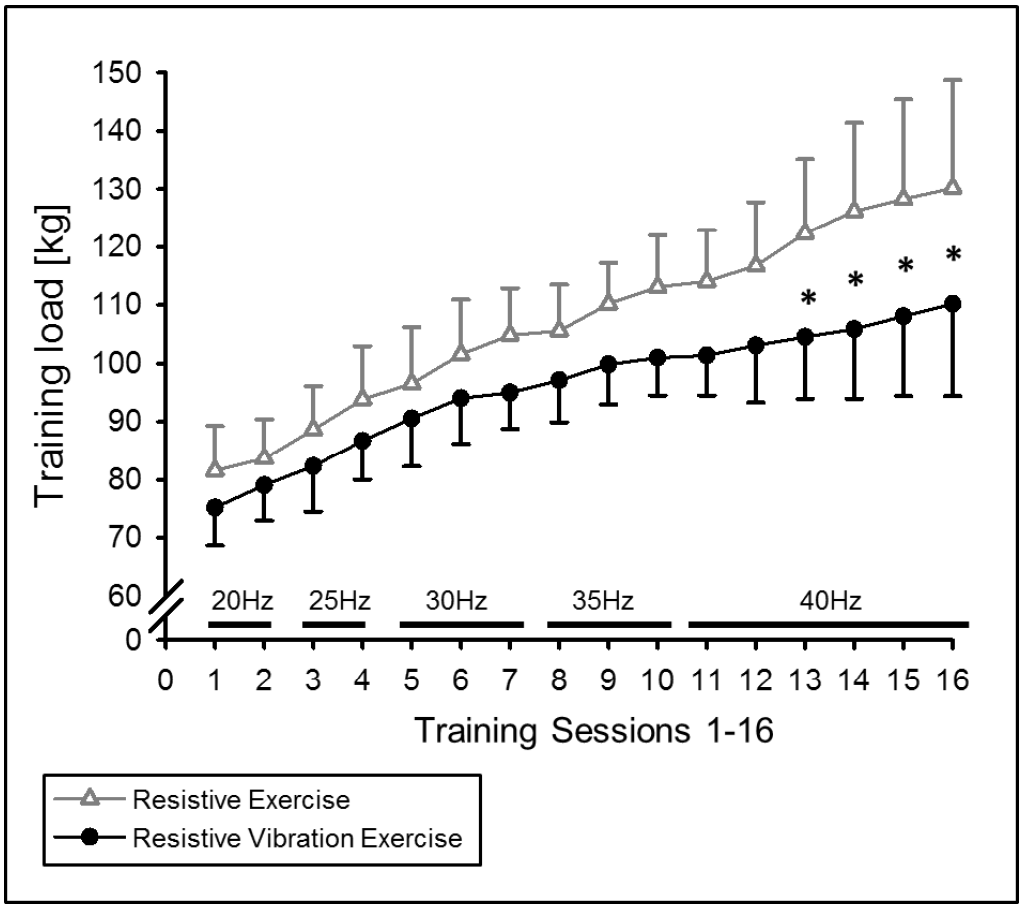

Figure 5. Training load increase during the 6-week training intervention. Bars indicating 20-40Hz refer to the applied vibration frequency in the RVE group. Training loads increased over time in both groups (time effect: P<0.001). The training load increase was more pronounced in the Resistive Exercise group and after the 13th training session, the RE group trained with significantly higher training loads (*P<0.01).

Rating of Perceived Exertion (RPE)

The perceived exertion of the initial training was rated as “hard” according to the Borg RPE scale, and there was no difference between groups: 15.5 ± 1.6 (RE) vs. 15.9 ± 1.3 (RVE), *P* = 0.52, see Figure 6. RPE data derived during the 6-week training reveal that superimposed vibrations did not alter RPE as there was no significant group effect (*P* = 0.73). However, there was an overall increase in RPE over time (*P* = 0.048). Post-hoc analyses showed that the RPE was higher during training 9-16 when compared to training 1-4 (*P* < 0.05). During the last training, RPE accounted for 15.9 in the RE group and 16.38 in the RVE group. Of note, RPE during the last training was comparable between groups (*P* = 0.15), although the RE group trained with significantly higher training loads (*P* = 0.003). Furthermore, there was no correlation between RPE and heart rate (R= -0.13; R2 = 0.017; *P* = 0.42) as previously described for endurance exercise[16].

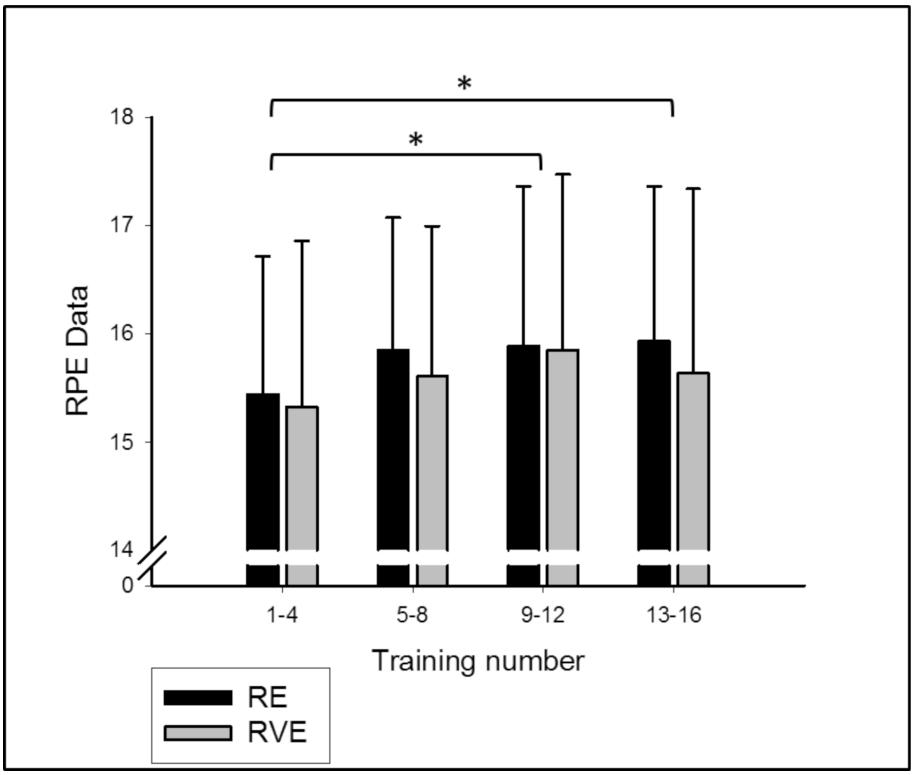

Figure 6. Rating of the training's perceived exertion. The subjects in both groups rated the perceived exertion (RPE) of the training to "hard" and there was no difference between the Resistive Exercise (RE) and Resistive Vibration Exercise (RVE) groups. RPE was significantly higher in trainings 9-16 compared to trainings 1-4 (*$P < 0.05$).

Cardiovascular parameters at rest

Resting Systolic Blood Pressure (SBP) and Diastolic Blood (DBP) pressure were both decreased from pre levels during the follow-up measurement after 6 weeks of training (SBP: $P = 0.003$; DBP: $P = 0.001$) with no significant differences between the two groups (SBP: $P = 0.06$; DBP: $P = 0.5$) as depicted in Table 3. Post-hoc analyses revealed that the decrease of DBP was more pronounced in the RVE group as this group depicted significant decreases ($P = 0.01$), whereas the decrease of DBP did not reach significance in the RE group ($P = 0.055$). Resting heart rate (HR) remained unaffected by the training intervention in both groups ($P = 0.14$), see Table 3.

	Rest				During Training			
Group	**RE**		**RVE**		**RE**		**RVE**	
Variable	**Pre**	**Post**	**Pre**	**Post**	**Initial**	**Final**	**Initial**	**Final**
SBP [mmHg]	126 ± 8	118 ± 11**	122 ± 4	113 ± 9**	147 ± 18	143 ± 13	147 ± 13	142 ± 18
DBP [mmHg]	71 ± 9	65 ± 11 **	71 ± 6	62 ± 8**	81 ±8	72 ± 9 ***	82 ± 7	74 ± 10***
HR [bpm]	55 ± 9	52 ± 7	56 ± 8	54 ± 7	125± 17	127 ± 15	126 ± 21	131 ± 23

Table 3. Cardiovascular parameters at rest (left) and during exercise (right). Stars indicate significant difference (time effect) within the same group: *P<0.05; **P<0.01; ***P<0.001. Pre and Post refer to resting values before and after 6 weeks of training; Initial and Final Training refer to the first and last exercise session of the 6-week training intervention. SBP: systolic blood pressure; DBP: diastolic blood pressure; HR: heart rate.

Cardiovascular parameters during exercise

Blood pressure and heart rate measured within single training sessions were comparable between sets ($P > 0.28$) and therefore, data of the three sets were pooled for further analysis. There was a trend of decreased systolic blood pressure during exercise after 6 weeks of training in both groups, which however failed to reach the level of significance ($P = 0.052$). Diastolic blood pressure during exercise was significantly decreased in both groups ($P < 0.001$). As a result of the decreased DBP with unaltered SBP, exercise pulse pressure (=SBP-DBP) was significantly increased in both groups after 6 weeks of training ($P = 0.04$). Six weeks of training did not alter exercise heart rate in neither of the groups ($P = 0.39$), see Tab.3. Exercise blood pressure and exercise heart rate did not differ when comparing RE to RVE (SBP: $P = 0.9$; DBP: $P = 0.6$; HR: $P = 0.5$).

DISCUSSION

Feasibility

The incremental design of the training was reflected by an increase in Borg RPE over time (Fig. 6), as the training was perceived as significantly “harder” in training sessions 9-16 compared to training sessions 1-4. The subject’s daily physical activities were comparable between the two groups and did not change over the duration of the study (Freiburg Questionnaire). These data indicate that the obtained results from the EVE study actually derive from the training intervention itself and were not induced by external factors.

While vibration frequency was increased on a weekly basis, the RVE group trained at equal or higher training loads compared to the previous week. Only in four cases out of 52 individual increases in vibration frequency (= 4 frequency increases * 13 subjects), training loads had to be decreased due to an increase in vibration frequency when training with frequencies above 35Hz. When training with frequencies between 20 and 30Hz, superimposed vibrations were well tolerated. However, data from the present study suggest that the risk of low back pain is substantially increased when performing resistance exercise with superimposed vibrations and frequencies above 30Hz (see Tab.2). Seven out of thirteen subjects that concluded the study successfully complained about low back pain, which would probably be classed as uncomfortable, but not severe. The back pain might either derive from the vibration itself, or from the way that the guided bar-bell was employed, which was always with a certain reclination toward the back. This could have increased the amount of instability in the movement when training with high vibration stimulation. This lack of stability might have caused the training incident that led to the drop-out of one subject in the RVE group. However, it remains unknown whether the vibration component was actually the cause for the training incident.

Demands

Increase of training load with and without superimposed vibrations

There was no difference between the two groups concerning One-Repetition Maximum or jump height at the beginning of the study, indicating two groups with comparable muscular performances. As expected, training loads were increased over time. However, after the 13th training session, when RVE subjects trained with 40Hz simultaneous vibrations, the increase of training weight was hampered (Fig. 5) compared to the group training without vibrations. In the end of the study, the RE group trained at 18% higher training loads compared to the RVE group. It is known that sinusoidal vibrations engender increases in peak foot acceleration to the power of two [10], and thus, increases in vibration frequency lead to pronounced elevations of musculoskeletal forces. We conclude from our data that the increase of training weight (external training load) might be hampered by vibration-induced elevation of musculoskeletal forces (internal training load) and the combination of the two add up to the total muscle loading during RVE. This idea is supported by the Rating of Perceived Exertion data, which indicate that training at lower weights with 40Hz WBV was perceived equally demanding as training without vibrations and higher weights.

Chronic cardiovascular adaptations at rest

There is strong evidence supporting beneficial effects of endurance exercise upon cardiovascular health such as decreases in blood pressure and heart rate [1,2]. However, limited data are available on the effect of long-term resistance exercise training in healthy, recreationally active people. Resistance exercise has been reported to have beneficial effects in obese subjects as well as in people with metabolic syndrome or hypertension [17–19]. Previous studies involving healthy young males show that resting systolic and diastolic blood pressures were decreased by a resistance training intervention [8,20]. Another study shows a 4% decrease in resting systolic with no change in diastolic blood pressure [5]. Results from the present study show that resting systolic and diastolic blood pressures were both decreased by 7 to 12 % after only six weeks of training and there were no alterations in resting heart rate. Our data support the view that high-resistance exercise is beneficial for cardiovascular health. Further, our data suggest that superimposed vibrations might be additionally beneficial as diastolic blood pressure was significantly decreased only in the RVE group.

Chronic adaptations of the acute cardiovascular responses to resistance exercise

It has been shown that body builders have lower systolic and diastolic blood pressures and heart rates during resistance exercise compared to recreationally active people [21]. Previous studies have reported that resistance training results in adaptations that hamper the acute training-induced increases in heart rate and blood pressure [22,23]. In the current study, we found that 6 weeks of resistive exercise decreased diastolic blood pressure during exercise whereas systolic blood pressure and heart rate were unaltered compared to the initial training. This decrease in diastolic blood pressure might derive from increased vasodilation during exercise and thus, the applied training interventions in the current study seem to have improved vascular responsiveness. This idea is supported by previous studies showing that WBV increases blood flow velocity after vibration termination [24,25], indicating vibration-induced dilation of feeding arteries. Our data reveal that only exercising diastolic blood pressure was decreased after 6 weeks of training, whereas systolic blood pressure remained unaltered, yielding increases in pulse pressure (=SBP-DBP). As pulse pressure is known to be proportional to stroke volume [26], there is evidence that the resistive exercise intervention conducted in this study increased cardiac stroke volume and

maybe cardiac output. There was, however, no additional effect of superimposed vibrations, neither during the first training nor after 6 weeks of training.

Summary and Conclusion

In summary, both training interventions were feasible and the incremental training design was reflected by an increase in RPE. Superposition of vibrations to resistive exercise for some reasons hampered the increase of training load when training at frequencies above 35Hz. Furthermore, our data show that 6 weeks of resistance exercise decreased resting blood pressure (systolic and diastolic) as well as exercising diastolic blood pressure. We conclude that WBV in combination with high-resistance exercise is well tolerated when training with frequencies below 35Hz. However, when training with 35Hz and above, this exercise type seems to foster back pain and to reduce training performance. It is possible that training with side-alternating vibration above 30 or 35Hz may elicit sub-optimal results. Thus, it might not be recommendable to use these high frequencies combined with resistance exercise, at least not for non-athletes. Finally, our data also demonstrate a beneficial effect upon arterial blood pressure.

Acknowledgements

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4.2 Paper 2: 'Whole-body vibrations do not elevate the angiogenic stimulus when applied during resistance exercise'

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Abstract

Knowledge about biological factors involved in exercise-induced angiogenesis is to date still scanty. The present study aimed to investigate the angiogenic stimulus of resistance exercise with and without superimposed whole-body vibrations. Responses to the exercise regimen before and after a 6-week training intervention were investigated in twenty-six healthy male subjects. Serum was collected at the initial and final exercise sessions and circulating levels of matrix metalloproteinases (MMP) -2 and -9, Vascular Endothelial Growth Factor (VEGF) and endostatin were determined via ELISA. Furthermore, we studied the proliferative effect of serum-treated human umbilical vein endothelial cells in vitro via BrdU-incorporation assay. It was found that circulating MMP-2, MMP-9, VEGF and endostatin levels were significantly elevated ($P<0.001$) from resting levels after both exercise interventions, with higher post-exercise VEGF concentrations in the resistance exercise (RE) group compared to the resistive vibration exercise (RVE) group. Moreover, RE provoked increased endothelial cell proliferation in vitro and higher post-exercise circulating endostatin concentrations after 6 weeks of training. These effects were elusive in the RVE group. The present findings suggest that resistance exercise leads to a transient rise in circulating angiogenic factors and superimposing vibrations to this exercise type might not further trigger a potential signaling of angiogenic stimulation in skeletal muscle.

INTRODUCTION:

There is growing evidence that physical activity is a potent stimulator of angiogenesis in skeletal and cardiac muscle [1]. Endurance training is thought to increase capillarity in skeletal muscle [2], whereas high resistance training has been shown to decrease capillary density [3], most likely as a result of fibre hypertrophy with insufficient angiogenesis. Knowledge about the exact mechanisms of blood vessel growth is to date still scanty. In the current models of sprouting angiogenesis, capillary formation involves two essential steps, namely (i) degradation of the extracellular matrix (ECM) surrounding the capillary and (ii) activation, migration and proliferation of capillary endothelial cells [4].

ECM breakdown is mediated by a family of zinc- and calcium-dependent enzymes, the matrix metalloproteinases (MMP) [5]. The proteases MT1-MMP, MMP-2 and -9 seem to play a crucial role in the formation of new capillaries in skeletal muscle [6] and previous studies reveal that their serum concentrations are significantly elevated after endurance exercise [7]. Furthermore, members of the MMP-family are known to release endostatin by proteolytic cleavage of the C-terminal NC1 domain of Collagen XVIII [8]. To date, the role of endostatin in the angiogenic process is not clear due to its complex signaling functions. As both pro-angiogenic [9] and anti-angiogenic [10] characteristics have been described for endostatin, it has been considered to function as an angiogenic modulator [11]. Endostatin seems to play a crucial role in exercise-induced angiogenesis, as serum concentrations were acutely elevated after endurance exercise [12,13]. However, other studies have reported decreased serum concentrations of endostatin as an adaptation to long-term endurance training [7].

Endothelial cell activation, migration, and proliferation is mediated by Vascular Endothelial Growth Factor (VEGF), a potent endothelial cell mitogen [14]. VEGF has been shown to be activated upon elevated shear stress perturbation [15], muscle stretch [16] and hypoxia [17]. Additionally, VEGF has been reported to be essential for exercise-induced angiogenesis in skeletal muscle [18]. The findings of a previous study evaluating the effects of endurance exercise with and without whole-body vibrations revealed that circulating VEGF was specifically increased in the group where vibrations were superimposed to the exercise stimulus [13].Of note, it has been suggested that the mechanical stimulus of whole-body

vibration (WBV) increases shear stress at the walls of blood vessels [19], leads to increases in blood flow velocity after vibration termination [20] and can elicit muscle de-oxygenation [21]. Based on the finding that shear stress and hypoxia are able to induce angiogenesis [4], we hypothesized that the superposition of a vibration stimulus to resistance exercise would add a pro-angiogenic stimulus to the exercise. It would be desirable to find a novel training mode that concurrently increases muscle strength and induces capillary growth to optimize the flux of oxygen and nutrients to the muscle and thus improve muscular performance. In order to investigate the pro-angiogenic stimulus of the exercises, we determined serum concentrations of the angiogenic factors MMP-2, MMP-9, VEGF and endostatin at rest and in response to resistance exercise and resistive vibration exercise. Additionally, we performed in vitro assays to evaluate the proliferative property of exercise-serum treated endothelial cells.

MATERIAL AND METHODS

Ethics statement

Twenty-six healthy, recreationally active male subjects (26±0.8 years) were included into the study after providing a written informed consent. The study was conducted in compliance with the Declaration of Helsinki following approval by the Ethics Committee of the Northern Rhine medical association (Ärztekammer Nordrhein) in Düsseldorf (application no. 2010-174).

Study design and subject characteristics

The present EVE study ("molecular and functional Effects of Vibration Exercise") was conducted in a stratified, randomized two-group parallel design. A detailed description of the exercises and study design has been published elsewhere [22]. Any competitive sports, participation in strength training during the past six months, smoking, diabetes as well as any current medication were considered as exclusion criteria. Subjects were stratified into two matched groups according to their maximum jumping height, forming two groups with comparable neuromuscular fitness [23]. A coin was then tossed to randomly assign the groups to one of the two training interventions: resistance exercise or resistive vibration exercise. The subjects anthropometric data at baseline are given in Table 1, and no statistically significant group difference was found ($P > 0.11$).

	RE group (n = 13)	**RVE group (n = 13)**	***P* - value**
Age [yrs]	23.4 (± 1.4)	24.3 (± 3.3)	0.52
Body mass [kg]	75.0 (± 4.7)	74.7 (± 6.9)	0.08
Height [m]	1.79 (± 0.05)	1.8 (± 0.1)	0.31
BMI	23.4 (± 1.4)	23.5 (± 2.1)	0.11
CMJ height [cm]	42.2 (± 4.6)	41.7 (± 2.2)	0.97
Maximal performance on cycle ergometer test [W / kg body weight]	3.3 (± 0.3)	3.3 (± 0.4)	1.00

Table 1. Anthropometric data of EVE subjects at baseline. BMI: Body Mass Index., CMJ: Counter movement jump. There was no difference between the two groups. Values are means ± SD

Training design

The present study was designed to compare acute and long-term effects of two training interventions: resistance exercise (RE) and resistive vibration exercise (RVE). Participants trained 2-3 times per week for six weeks (completing 16 exercise sessions), with each session lasting 9min. Participants trained with weights on a guided barbell (PTS Dual action Smith, Hoist, U.S.A). The individual training load was set at 80% of their One-Repetition-Maximum (1-RM), which was determined according to the method described by Baechle and Earle [24]. The exercise consisted of squats (with each 2 sec. eccentric and 2 sec. concentric phase) and heel raises (with each 1 sec. eccentric and 1sec. concentric phase), divided by a 1-min break. Movement rhythm was guided by a metronome. Each exercise session consisted of a warm-up composed of two sets with each 10 squats and 15 heel raises with the unloaded barbell (15kg) as training weight. The actual exercise was carried out in three sets: first and second sets were composed of 8 squats (= 32sec. per set) and 12 calf raises (=24sec. per set) and in the third set, maximum number of repetitions for squats and calf raises were performed. The subjects in the RVE group performed the resistance exercise protocol with simultaneous side-alternating whole-body vibrations (Galileo® Fitness, Novotech, Germany) with a 6mm peak-to peak displacement, whereas subjects in the RE group trained with the same setting, without superimposed vibrations.

The training followed an incremental training design with regards to weight and vibration frequency. Training weights were increased over time according to the subjects' individual training progressions, as described previously [22]. In brief, the number of squats in the 3rd set was used as a reference to re-determine the subjects' individual 80% of the 1-RM for the following training, using the method described by Baechle and Earle [24]. Training weights in the RE group increased from 75.2 ± 1.8kg during the initial exercise to 130.2 ± 5.1kg during the final exercise. Weight increase was significantly smaller the RVE group, which increased from 81.5 ± 2.1kg during the initial exercise to 110.2 ± 4.4kg during the final exercise. Training weight increase was hampered by training with vibration frequencies above 35Hz, as discussed in the methodological paper on the training design previously published [22].
Vibration frequencies were increased from 20Hz in the first week to 40Hz during the last two weeks with 5-Hz weekly increments. The reason for the increase in vibration frequency was that we aimed to test physiological responses when exercising at 40Hz side-alternating WBV, which to the best of our knowledge has not been tested in any other study. Pilot testing revealed that resistance exercise with 40Hz side-alternating WBV is more challenging for people not accustomed to WBV, suggesting that it could potentially elicit greater effects than lower vibration frequencies, but also that one must envision problems when embarking directly on such high a frequency. Thus, in order to prevent problem-related drop-out from the RVE group and thus a study bias, we decided to initially set the vibration frequency to 20Hz and to gradually increase the vibration frequency to 40Hz.

Serum collection

Venous blood samples were collected at the initial and final exercise sessions of the 6-week training intervention as illustrated in Figure 1. On that day, subjects had a standardised breakfast (two wheat bread rolls with butter and jam) two hours before exercise. Blood was collected one hour prior to exercise (Rest) and +2min, +5min, +15min, +35min and +75min after exercise through a short catheter into serum monovettes (Sarstedt, Nümbrecht, Germany) from the cephalic vein, allowed to clot for 10 minutes, centrifuged at 3000 rpm at 4°C (Heraeus Multifuge 1S-R, Thermo Scientific, Waltham, MA, USA), distributed into small tubes and immediately frozen at -20°C until analysis.

ELISA analyses

Serum levels of MMP-2 (free pro- and active MMP-2 [ng/ml]), MMP-9 (92 kDa pro- MMP-9 and 82 kDa active MMP-9 isoforms [ng/mL]), VEGF (total VEGF, [pg/ml]) and endostatin (total endostatin, [ng/ml]) were detected in double determinations using Enzyme-linked Immunosorbent Assay (ELISA) kits (R&D Systems, Wiesbaden, Germany) according to the manufacturer's instructions.

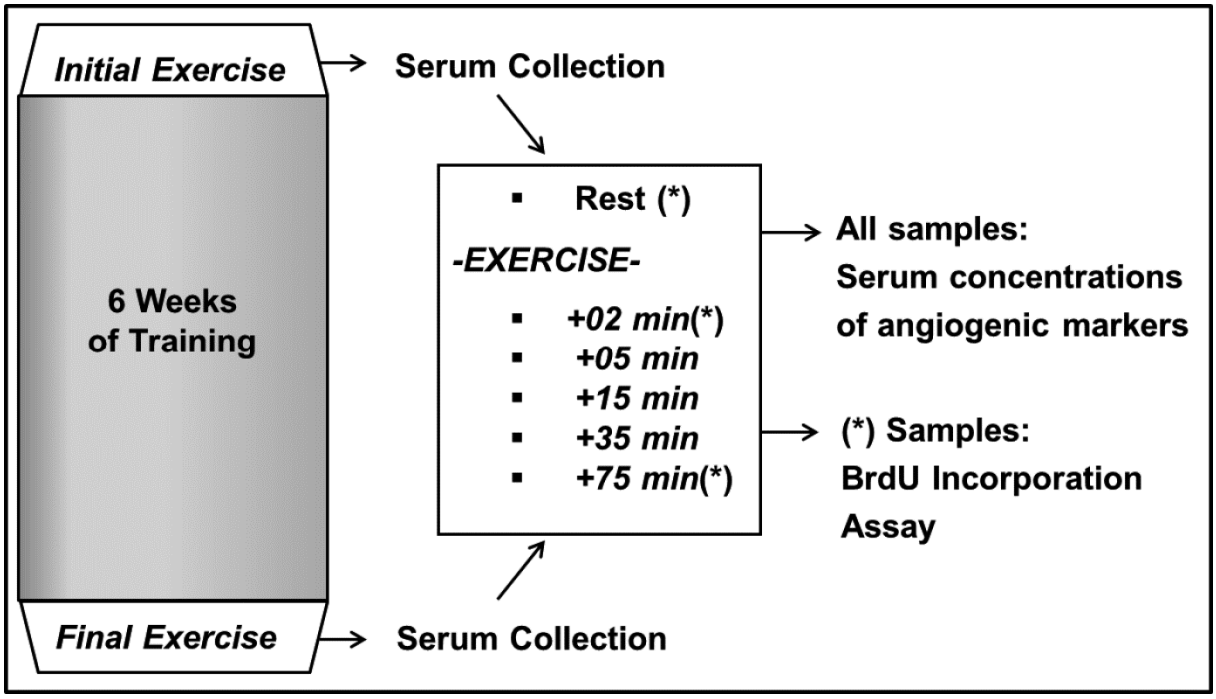

Figure 1: Study Design. Serum was collected at the initial and final exercise sessions of a 6-week training intervention. Time points of serum collection were 1 hour prior to exercise (Rest) and 2, 5, 15, 35 and 75 minutes after exercise termination. Serum concentrations of angiogenic markers (MMP-2, MMP-9, VEGF and endostatin) were determined for all serum samples, BrdU incorporation assay was performed with the serum samples indicated with (*): Rest, +2min and +75min.

Cell lines and culture conditions

Human Umbilical Vein Endothelial Cells (HUVEC, #C-12200, PromoCell, Heidelberg, Germany) were cultured at 37°C and 5% CO_2 in basal medium with added growth supplements (Endothelial Cell Growth Medium KIT, #C-22110, PromoCell, Heidelberg, Germany). Prior to incubation with human serum and 5-Bromo-2-Deoxyuridine (BrdU), cells were split into 96-well plates (Detach Kit, #C-41210, PromoCell, Heidelberg, Germany) and cultured in starvation medium (i.e. basal medium with only 0.5% Fetal Calf Serum as growth supplement) for 24 hours. BrdU incubation was performed in conditioned medium (i.e. basal medium containing 2% of human serum providing growth and proliferation factors). Sera

obtained from pre- and post-training (Rest, *+2min* and *+75min* post) at each initial and final exercise sessions were used for generating the conditioned medium, see Figure 1.

BrdU incorporation assay

Samples were incubated with BrdU for 20 hours and detection of BrdU incorporation was performed in double determinations via ELISA (BrdU Cell Proliferation Assay Kit, #6813, Cell Signaling Technology, Danvers, MA, USA) according to the manufacturer's instructions.

Statistical Analyses

Statistical analyses were performed using STATISTICA 10 for Windows (Statsoft, Tulsa, Oklahoma, USA, 1984-2010). The acute effect of either resistive exercise (RE) or resistive vibration exercise (RVE) on serum concentrations of the angiogenic factors MMP-2, MMP-9, VEGF and endostatin was determined via repeated measures ANOVA with time (Rest *vs. +2min*, *+5min*, *+15min*, *+35min*, *+75min* after training) and training status (initial *vs.* final training session) as factors. BrdU incorporation data were normalised to fold increases from resting levels (i.e. absorption of cells incubated with serum derived *+2min* and *+75min* after exercise divided by absorption of cells incubated with serum at *Rest*). A repeated ANOVA was performed with time (*+2min vs. +75min*) and training status (initial *vs.* final training) as factors. Tukey's test was used for post-hoc testing. Values are given as means ± SEM. Statistical significance level was set at $P < 0.05$.

RESULTS

Resting levels

Resting levels of the circulating angiogenic factors MMP-9, VEGF and endostatin were comparable before and after the 6-week training intervention ($P > 0.19$) and there were no significant differences in resting levels between the two groups ($P > 0.68$), as shown in Table 2. Resting levels of MMP-2 measured at the final exercise session differed between groups with the RVE group depicting higher values than the RE group (RVE: 193.0±8.71ng/mL *vs*. RE: 172.0±8.5ng/mL, $P < 0.001$), which had not been the case at the initial exercise session ($P = 0.37$).

	RE		RVE	
	Initial exercise	**Final exercise**	**Initial exercise**	**Final exercise**
MMP-2 [ng/ml]	181 ± 9	172 ± 8	186 ± 6	193 ± 8$^{###}$
MMP-9 [ng/ml]	231 ± 17	218 ± 19	203 ± 21	224 ± 35
VEGF [pg/ml]	234 ± 53	242 ± 50	211 ± 37	216 ± 38
Endostatin [ng/ml]	102 ± 4	99 ± 5	105 ± 3	103 ± 4

Table 2. Resting levels of angiogenic markers measured at the initial and final exercise sessions of the 6-week training intervention. $^{###}$Significant difference ($P < 0.001$) between the groups at the final exercise. RE: resistance exercise, RVE resistive vibration exercise MMP: Matrix metalloproteinase, VEGF: Vascular Endothelial Growth Factor. Values are means ± SEM.

Effect of Resistance Exercise upon angiogenic factors

MMP-2, MMP-9, VEGF and endostatin were all significantly increased from resting levels after both resistive exercise and resistive vibration exercise (time effect: $P < 0.001$) and all factors depicted maximum concentrations two minutes after exercise termination. In the following, relative increases from resting levels are given for the maximum concentrations that were measured at the time point *+2min*.

MMP-2

Acute effect: In the RE group, MMP-2 levels were increased from resting levels by 8% (SEM = 2%, $P = 0.001$) two minutes after the initial training and decreased by 5% (SEM = 1%, $P = 0.035$) at the time point *+75min*. In the RVE group, on the contrary, MMP-2 levels were not significantly elevated from resting levels after the initial training ($P = 0.9$), and were decreased by 8% (SEM = 2%, $P = 0.01$) at the time point *+75min* (Fig. 2A). There were no significant differences between RE and RVE groups at the initial training ($P = 0.99$).

Long-term effect: In the RE group, there were no significant differences in the time courses when comparing initial and final training sessions ($P = 0.99$) as depicted in Fig. 2B(i). At the final training of the RVE group, however, the acute MMP-2 response was generally elevated over the time course of the initial training (time*intervention effect: $P = 0.049$), see Figure 4B(ii). Post-Hoc testing revealed that MMP-2 concentrations were significantly higher at the time points *+2min* ($P = 0.028$), *+15min* ($P = 0.019$) and *+75min* ($P = 0.015$) in the RVE group compared to the same time point at the initial training. While MMP-2 was not elevated from resting levels in the RVE group after the initial training of the 6-week training intervention,

MMP-2 concentrations were significantly elevated by 8% (SEM = 2%, $P = 0.02$) two minutes after the final training. Due to the RVE-specific increases in MMP-2 concentrations, clear group differences were apparent at the final exercise session with the RVE group depicting significantly higher MMP-2 concentrations compared to the RE group at rest and after exercise (RE *vs.* RVE: $P < 0.01$).

MMP-9

Acute effects: MMP-9 was elevated from resting levels 2-15min after exercise (time effect: $P < 0.001$). The MMP-9 increase after the initial exercise accounted for 71±19% in the RE group and 74±16%) in the RVE group with no significant differences between groups (RE *vs.* RVE: initial exercise: $P = 0.439$; final exercise: $P = 0.35$), see Fig. 3A.

Long-term effects: There was no effect of the 6-week training intervention upon the acute MMP-9 response in serum (initial *vs.* final exercise: RE: $P = 0.44$; RVE: $P = 0.98$), see Figure 3B.

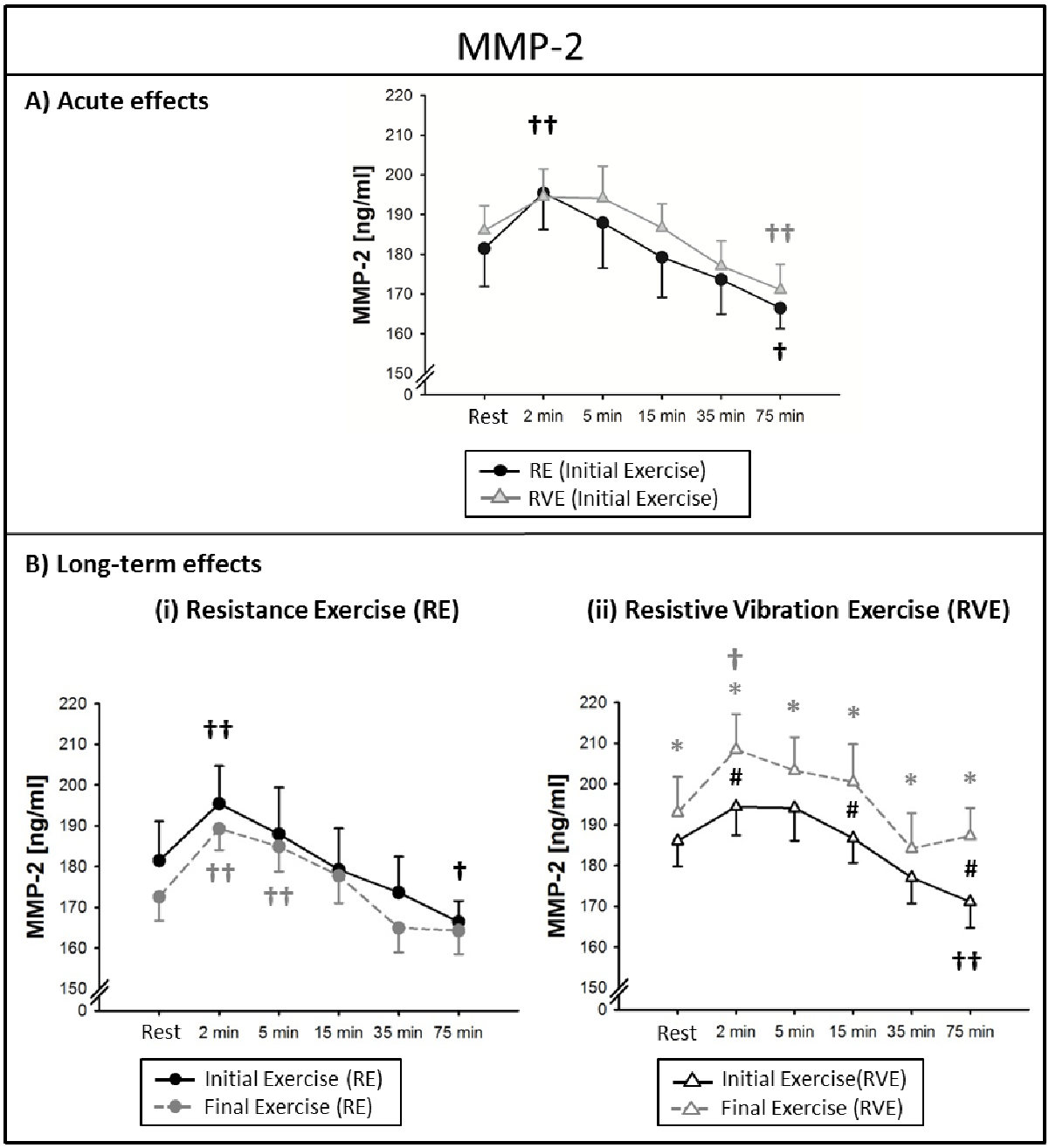

Figure 2: Circulating matrix metalloproteinase (MMP)-2 levels at rest and 2-75 min after exercise. Data points indicate mean serum concentrations (± SEM) at the initial and final exercise sessions of a 6-week training intervention (n = 13). Significant differences from resting levels (time effect): †$P < 0.05$, ††$P < 0.001$; significant differences from the initial exercise at the same time point $^{\#}P < 0.05$; significant differences between groups at the final exercise $^{*}P < 0.01$. (A) Acute effects of resistance exercise (RE) and resistive vibration exercise (RVE): MMP-2 was elevated from resting levels only in the RE group. (B) Long-term effects: the acute response after the final exercise in the RVE group was elevated over the time course measured at the initial exercise and the RVE group depicted significantly higher MMP-2 levels at all time points compared to the RE group.

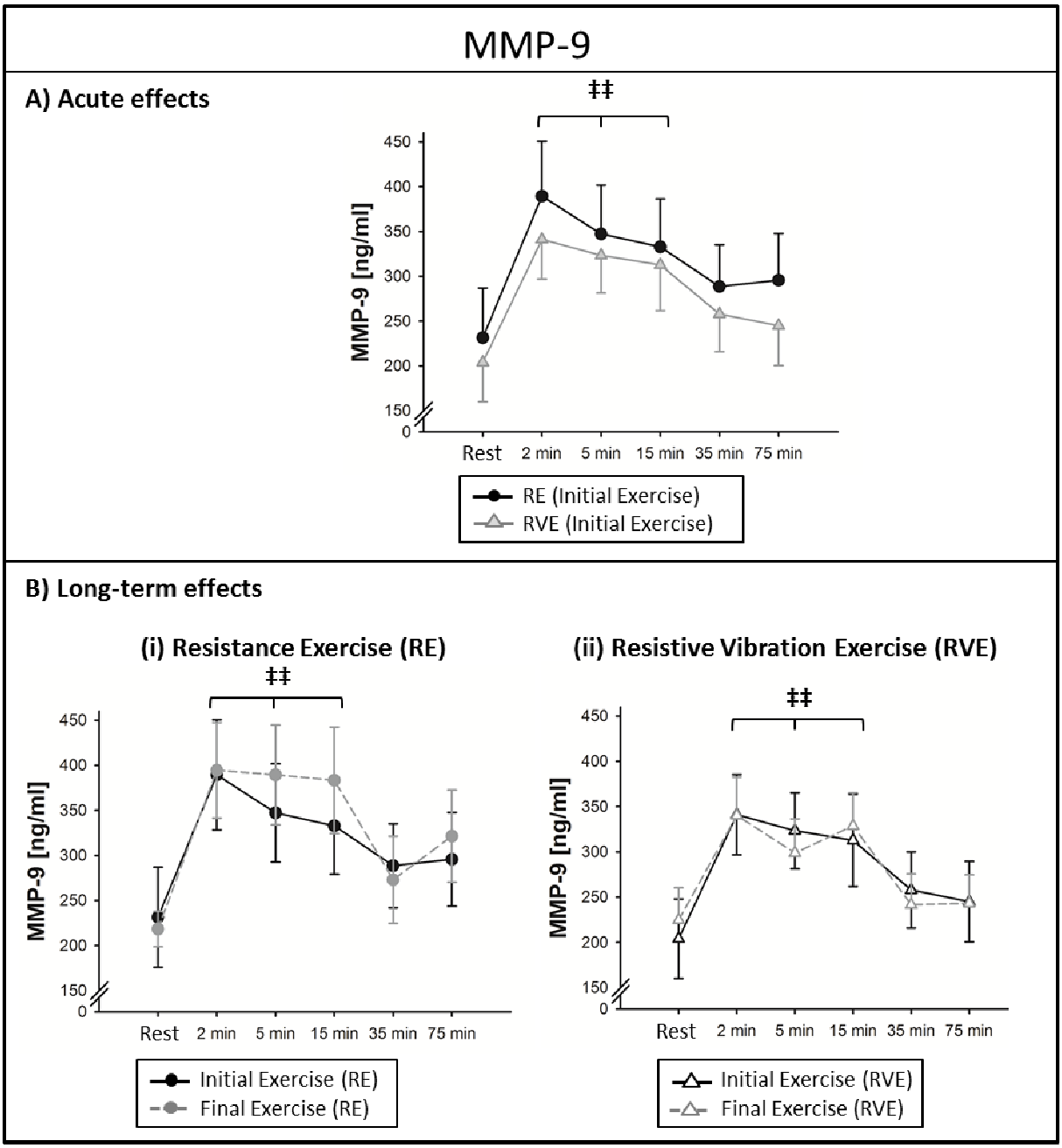

Figure 3: Circulating matrix metalloproteinase (MMP)-9 levels at rest and 2-75 min after exercise. Data points indicate mean serum concentrations (± SEM) at the initial and final exercise sessions of a 6-week training intervention (n = 13). (A) Acute effects of resistance exercise (RE) and resistive vibration exercise (RVE); (B) Long-term effects: In both groups, MMP-9 levels were increased over resting levels 2-15 min after exercise. Significant differences from resting levels (time effect): $^{‡‡}P < 0.01$. There were no differences between initial and final exercises of the 6-week intervention in neither group.

Endostatin

Acute effects: Serum levels of endostatin were increased from resting levels 2-15min after both RE and RVE (time effect: $P < 0.001$). After the initial training, endostatin levels were elevated by 17±3% in the RE group and by 22±4% in the RVE group with no significant differences between groups *(*$P = 0.85$), see Figure 4A.

Long-term effects: After the final exercise, endostatin concentrations in the RE group were uniformly greater than concentrations after the initial exercise (time * intervention effect: $P < 0.001$, see Figure 4B(i). This long-term effect was not seen in the RVE group (time * intervention effect: $P = 0.991$), see Figure 4B(ii).

VEGF

Acute effects: In the RE group, VEGF was elevated from resting levels 2-15min after the initial exercise (time effect: $P < 0.001$). In the RVE group, the response differed as this group showed elevated VEGF concentrations only at the time point *+2min* (time effect: $P < 0.001$). VEGF concentrations were significantly higher in the RE group with a 41±16% increase from resting levels compared to the RVE group, which showed a 33±7% increase at the time point *+2min* ($P = 0.014$). Significantly higher VEGF concentrations in the RE group compared to the RVE were also detected at the remaining time points 5-75min after exercise termination (*P*-values between 0.02 and 0.004), see Figure 5A.

Long-term effects: There were nonsignificant changes in the responses to the exercises after 6 weeks of training, (initial *vs.* final exercise: RE: $P = 0.520$; RVE: $P = 0.814$, see Figure 5B) and VEGF concentrations after the final exercise were also higher in the RE group compared to the RVE group (RE vs. RVE: *P*- values between 0.01 and 0.005).

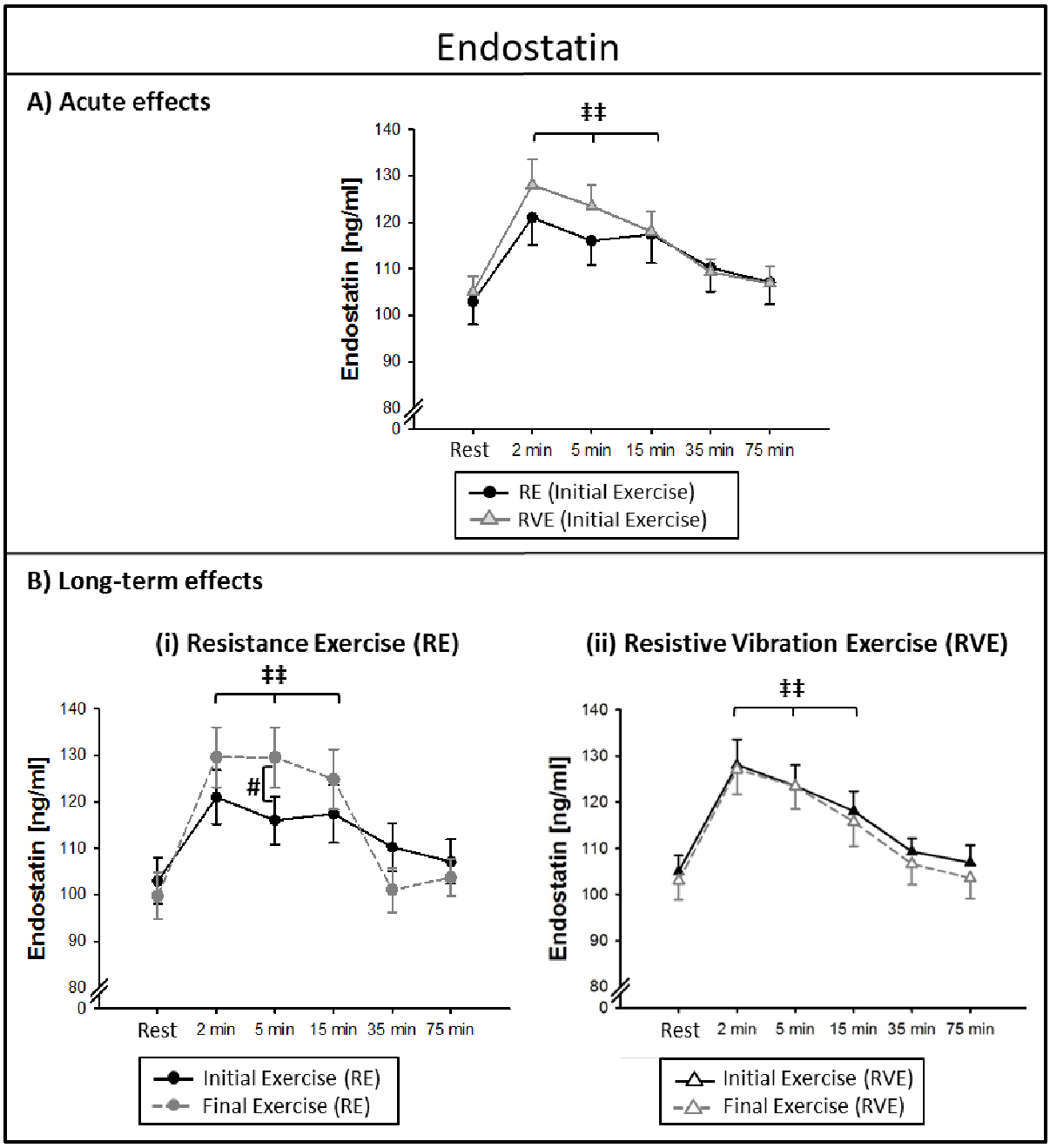

Figure 4: Circulating endostatin levels at rest and 2-75 min after exercise. Data points indicate mean serum concentrations (± SEM) at the initial and final exercise sessions of a 6-week training intervention (n = 13). Endostatin levels were increased over resting levels 2-15 min after training (time effect): $^{‡‡}P < 0.01$. (A) Acute effects of resistance exercise (RE) and resistive vibration exercise (RVE): the acute exercise effects did not differ between groups. (B) Long-term effects: circulating post-exercise endostatin levels in the RE group were higher at the final exercise compared to the initial exercise: $^{\#}P < 0.05$.

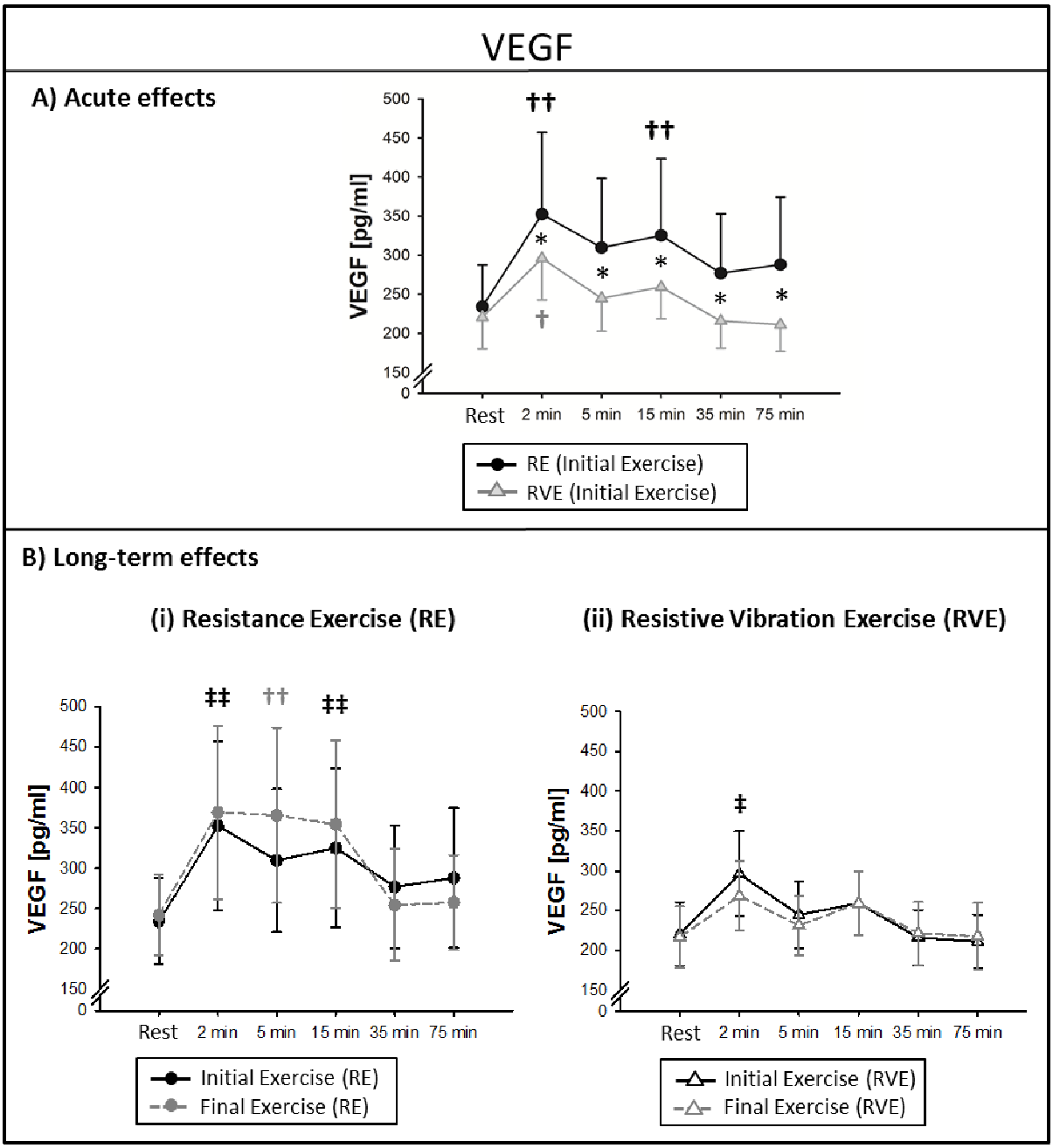

Figure 5: Circulating Vascular Endothelial Growth Factor (VEGF) levels at rest and 2-75 min after exercise. Data points indicate mean serum concentrations (± SEM) at the initial and final exercise sessions of a 6-week exercise intervention (n = 13). Significant differences from resting levels (time effect): †† $P < 0.01$; both indicated exercises: ‡‡ $P < 0.01$. (A) Acute effect of resistance exercise (RE) and resistive vibration exercise (RVE): VEGF was elevated from resting levels 2-15 min after RE and only 2 min after RVE with significantly higher VEGF levels in the RE group. (B) Long-term effects: there were no differences between initial and final exercises in neither group.

Endothelial Cell Proliferation

We used the human serum derived at rest and *+2min* and *+75min* after exercise to test the proliferative effect upon human umbilical vein endothelial cells (HUVEC) *in vitro*. These time points were suitable as the angiogenic factors measured via ELISA depicted maximum serum concentrations *+2min* after exercise termination and concentrations were back at resting levels at the time point *+75min*. Absorption data detecting BrdU incorporation were normalized to fold increases from resting levels.

Endothelial cells incubated with serum derived at *+2min* after resistance exercise showed an increased proliferation compared to cells incubated with *+75min* serum (time effect: P = 0.0171). This effect was not seen in the RVE group (time effect: P = 0.295). EC proliferation did not differ between cells treated with serum derived after the initial or final exercises in neither group (RE: P = 0.94; RVE: P = 0.91) and no significant differences between the groups were found (P = 0.122), see Figure 6.

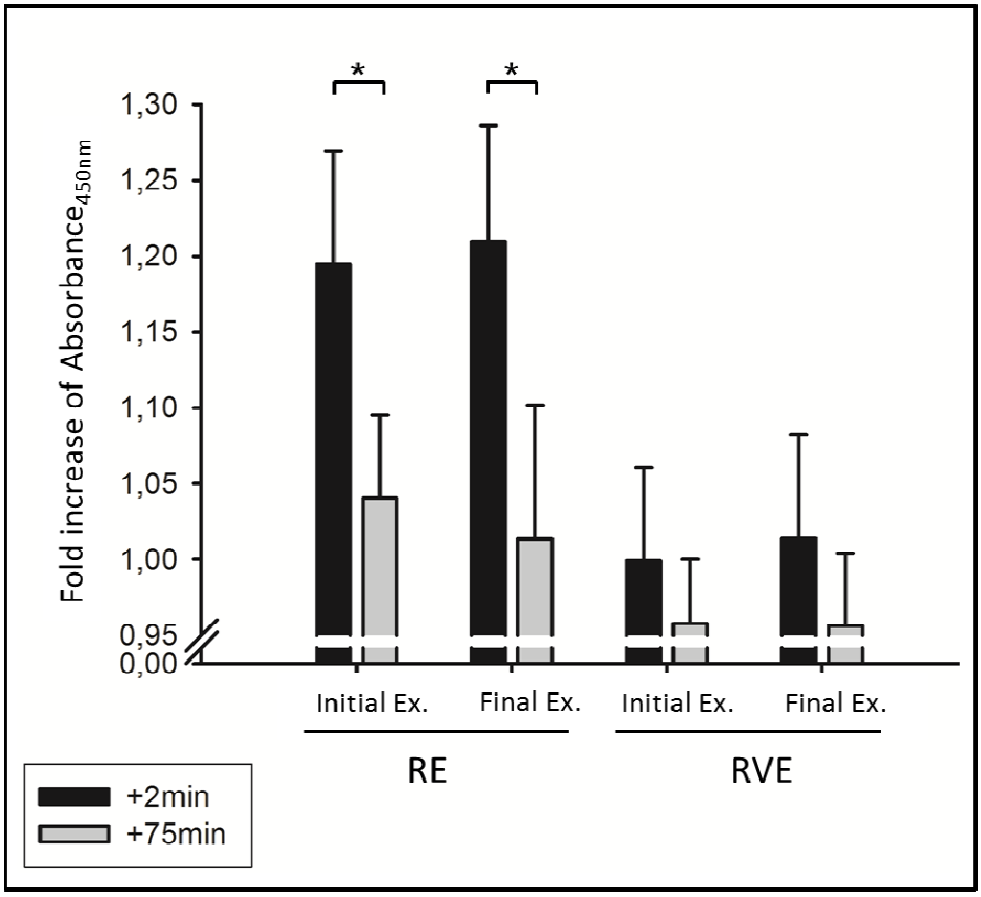

Figure 6: Endothelial cell proliferation measured via BrdU incorporation in human umbilical vein endothelial cells. Bars indicate fold increases of absorbance$_{450nm}$ of cells incubated with serum derived at rest compared with two minutes *(+2min*) and 75 minutes (*+75min*) after exercise. Initial Ex. / Final Ex.: initial and final exercises of a 6-week exercise intervention. RE: resistance exercise, RVE resistive vibration exercise. * time effect: P = 0.0171.

DISCUSSION

To test our hypothesis that superimposing whole-body vibrations to resistance exercise would add a pro-angiogenic stimulus to the training, we evaluated serum concentrations of angiogenic markers *in vivo* and their proliferative capacity upon endothelial cells *in vitro*. Our data indicate that resistance exercise leads to a transient increase of circulating angiogenic markers. Post-exercise serum concentrations of VEGF were higher in the resistance exercise (RE) group compared to the resistive vibration exercise (RVE) group. Additionally, the RE group provoked increased endothelial cell proliferation *in vitro* and showed higher exercise-induced endostatin concentrations. Both effects were elusive in the RVE group.

MMPs

Degradation of the vascular basement membrane is an initial step in angiogenic sprout formation and allows endothelial cells to migrate into the interstitial matrix in order to form a premature capillary lumen. Matrix metalloproteinases are crucial for extracellular matrix degradation and are thought to be essential for physiological angiogenesis [25]. MMPs have furthermore been implicated in the release and bioavailability of growth factors [26,27] and play a role in initiating endothelial cell migration and proliferation [28]. Our data show elevated serum MMP-2 levels two minutes after resistance exercise. In the group where whole-body vibrations were superimposed to the exercise, MMP-2 was not elevated after an initial exercise bout but showed an adaptation to long-term training; after 6 weeks of RVE, MMP-2 was elevated above resting levels and concentrations were higher (at rest and post-exercise) compared to the group that had trained without vibrations. This might be a compensatory adaptation to the initial lack of MMP-2. Beyond initiating capillary growth, MMP-2 has furthermore been shown to increase the bioavailability of insulin-like growth factor (IGF) through proteolysis of the IGF binding protein [26,27]. If this is the case, the observed increases in circulating levels of MMP-2 after six weeks of RVE might reflect an increased IGF-associated anabolic stimulation.

The presented MMP-9 data showed a prolonged increase compared to MMP-2 and MMP-9 was elevated from resting levels until 15min after both exercise regimes with no detectable long-term effect. A previous study measuring serum MMP-9 concentration pre and post eight weeks of resistance training *vs.* eight weeks of callisthenic training report an increase in the acute MMP-9 response after eight weeks of training only in the callisthenic group [29]. A study on downhill running showed no acute changes in serum MMP-2 but increased serum

MMP-9 levels immediately after exercise [30]. Running step tests increased plasma MMP-2 one hour post-exercise whereas plasma MMP-9 was increased immediately after exercise with decreased resting MMP-9 levels after six months of training [7].

Together with the present findings, these data suggest that MMP-responses to acute and long-term training interventions highly depend on workload, volume and contraction form of the exercise. The exposure to different mechanical stimuli seems to foster extracellular matrix remodeling in divergent ways, revealing a potential role of MMPs in initiating training-specific muscle adaptations. A limitation of the procedure is that the available antibodies do not differentiate between the active and pro-enzyme forms of MMPs and we did not measure serum concentrations of tissue inhibitors of metalloproteinases. Therefore, increased MMP-2 and MMP-9 immunoreactivity does not necessarily indicate an increased enzymatic activity.

Endostatin

Our data show that circulating endostatin was elevated from resting levels 2-15min after a bout of resistance exercise with no additional effect of superimposed vibrations. Previous studies report prolonged elevations of circulating endostatin compared to the time curves we observed: elevations in plasma from 1h [31] until 6h post exercise [12] have been reported after endurance exercise. After 90min of cycling exercise, Suhr and colleagues [13] found endostatin to be elevated in plasma 0-60min after exercise termination and superimposing vibrations to this exercise type shortened the elevation from baseline levels to 0min after exercise, which is an effect of superimposed vibration we did not observe in the present study.

Although we did not see any long-term adaptations in basal endostatin levels, as previously reported for endurance training [7], the response was altered after the 6-week training intervention. Endostatin concentrations in serum were acutely higher after 6 weeks of training and this adaptation was specific for the RE group. Thus, superimposed vibrations seemed to inhibit this biological adaptation to long-term training. Due to endostatin's complex signaling functions, it is not a simple task to interpret the physiological impact of elevated endostatin concentrations after exercise. Initially, endostatin was described as an anti-angiogenic protein [10] capable of inducing apoptotic signals in endothelial cells [32] and to inhibit EC migration, -proliferation and tube formation [33]. Conversely, it was later shown that endostatin has both pro- and anti-angiogenic functions depending on its

concentration and the proliferation status of endothelial cells [11]: towards the running opinion, Schmidt and colleagues [11] showed that endostatin concentrations of 50ng/mL induced EC proliferation and migration with no induction of apoptosis; whereas concentrations of 1000ng/mL and above had the contrary effect. Based on these data, the endostatin concentrations we reported in the present study (90-140ng/mL) lie close to the concentrations that were considered as a pro-angiogenic range. Thus, the observed increase in endostatin response after 6 weeks of training (RE only) might reflect a pro-angiogenic long-term training adaptation, which is inhibited by superimposed vibrations.

The acutely elevated endostatin levels seem to have a critical function during exercise. As recently demonstrated by our group, endostatin induces the release of the vasodilator NO in endothelial cells [34]. The acute exercise-dependent endostatin release therefore seems to be essential to activate signaling pathways that result in peripheral vasodilation and consequently improves oxygen delivery to working skeletal muscles to maintain the muscle performance capacity.

VEGF

The process of endothelial cell proliferation is mediated mainly by Vascular Endothelial Growth Factor (VEGF), a potent endothelial cell mitogen [14]. Exercise leads to increases of VEGF protein in muscle tissue [31] and VEGF has shown to be essential for exercise-induced angiogenesis in skeletal muscle [18]. VEGF serum concentrations were shown to be decreased [12,31] or elevated [35] after endurance-type exercise. Our data are to our knowledge the first that reveal acute increases of circulating VEGF immediately after resistance-type exercise. We could show that VEGF was elevated in serum 2-15 minutes after resistance exercise, whereas superposition of vibrations to the exercise shortened this response to only two minutes after exercise and provoked significantly lower VEGF concentrations compared to the group that trained without vibrations. As we did not measure VEGF expression in muscle tissue, this finding gives rise to multiple possible explanations. First, decreased circulating VEGF could indicate that more VEGF is still held and active in the tissue and has not been washed out into the blood. Second, reduced circulating VEGF upon vibration exposure could indicate that whole-body vibrations in some way prevented VEGF secretion or release in muscle tissue, which would indicate that superimposing vibrations would not be beneficial for a potential activation of angiogenic signaling in skeletal muscle. Third, VEGF is produced in many cell types and the increased

circulating VEGF might also derive from a systemic exercise effect which is not related to muscle tissue and could indicate enhanced endothelial regeneration, which would reflect a beneficial effect of resistance exercise that was inhibited by superimposed vibrations.

In a previous study in our lab, the effect of high-intensity cycling exercise with and without whole-body vibrations was evaluated and this study revealed contrary results considering vibration exposure: plasma VEGF levels were only increased in the group where vibrations were superimposed to the exercise stimulus [13]. As previous studies reveal that WBV increase the shear stress in blood vessels [19], Suhr and colleagues concluded that vibration-induced increases in shear stress-stimulated VEGF release as described by Milkiewicz and colleagues [15]. This explanation does not seem to be applicable in the present study, as our data reveal the contrary, i.e. reduced VEGF upon vibration exposure. Thus, whole-body vibration stimulation seems to have differential effects according to the mode it is applied. In the case of endurance cycling exercise, superimposed vibrations might be beneficial for promoting angiogenesis (reflected by increases in VEGF), whereas our data reveal that the contrary seems to be the case for resistance exercise. As exercise times in the aforementioned study (90min) were much longer compared to the present study (9min), it might well be that the initial effects of the exercises are comparable but the measured VEGF kinetics may differ due to the time shift in the measurements.

It is well known that levels of angiogenic markers differ according to the type of blood product in which they were measured (serum *vs.* plasma). Previous studies were inconsistent in the type of blood product used and this might contribute to discrepancies between studies.

Endothelial cell proliferation

One limitation of measuring angiogenic markers in serum is that their site of action resides within the muscle tissue itself and we determine merely the 'wash-out' in serum. Consequently, we sought to investigate whether and in which manner elevated serum concentrations would possibly influence endothelial cells *in vitro*, because this model is well-established to test general defined reactions of endothelial cells *in vitro* that might reflect *in vivo* situations.

As all factors showed maximum concentrations *+2min* after exercise and were back at resting levels *+75min* after exercise, we chose to treat human umbilical vein endothelial cells (HUVEC) with serum derived from these time points. We found that endothelial cells

incubated with serum derived *+2min* after RE showed increased proliferation compared to cells incubated with serum derived*+75min* after exercise. This effect was not seen in the RVE group. VEGF was the only angiogenic factor that showed group-specific differences after exercise (see Figure 5A). VEGF serum concentrations were higher *+2min* after RE ([352±104pg/mL] after initial- and [369±107pg/mL] after final exercise) compared to *+2min* after RVE ([280±50pg/mL] after initial- and [268±43pg/mL] after final exercise), which may be an explanation for the group-specific differences in cell proliferation. The recommended VEGF concentration for HUVEC culture is 500pg/mL (Endothelial Cell Growth Medium KIT, #C-22110, PromoCell, Heidelberg, Germany), which lie close to the VEGF concentrations we measured in the RE group. However, there are various additional factors that were not measured in the present study that, however, could have influenced HUVEC proliferation, i.e. basic Fibroblast Growth Factor [36], epidermal growth factor (EGF) or heparin-binding EGF-like growth factor [37].

Thus, our data, with certain limitations, reveal that superimposed whole-body vibrations to resistance exercise leads to decreased endothelial cell proliferation, probably due to decreased release or expression of VEGF. Considering long-term adaptations, we did not find any differences in HUVEC proliferation when comparing initial and final exercise sessions. Despite acutely higher endostatin levels during the final exercise in the RE group and higher MMP-2 concentrations in the RVE group, these effects were not reflected by increased cell proliferation during the final exercise.

Comparison of Time curves:

When comparing the time curves of MMP-9 with VEGF and endostatin, it seems that the exercise-induced increase of MMP-9 is paralleled by VEGF and endostatin. First, all factors were increased 2-15min after exercise and second, all three factors show increased mean concentrations after 6 weeks of training (although only significant for endostatin), see Figure 3B(i), 4B(i) and 5B(i). Conversely, the factor MMP-2 showed different kinetics as it was elevated only for two minutes after exercise and the long-term adaptation that was seen for MMP-2 in the RVE group was specific for MMP-2 and did not affect any of the other factors. In sum, these observations indicate that MMP-9, VEGF and endostatin seem to be interdependent, whereas MMP-2 seems to be differentially regulated. Our data are in line with previous observations in cell culture which showed that MMP's are capable of inducing VEGF release [38]. Moreover, the presented data confirm a previous finding in which the

authors described that MMP-9 was more prone to release VEGF compared to MMP-2 *in vitro* and that that MMP-2 regulation occurred independently of VEGF signaling [28]. The parallel increase of MMP-9 and endostatin confirms that endostatin is proteolytically released by MMP`s, as described previously [8] and our data hint to MMP-9 playing a larger part in this release compared to MMP-2, at least after bouts of resistance exercise.

Summary and Conclusion

In summary, our data show that RE leads to transient increases in circulating pro-angiogenic markers and furthermore, endothelial cell proliferation *in vitro* is increased by factors in serum obtained acutely after RE. Superimposing vibrations to resistance exercise decreases post-exercise circulating VEGF concentrations, which supposedly results in reduced endothelial cell proliferation *in vitro*. Six weeks of RE increased endostatin concentrations acutely after exercise, which is considered as a pro-angiogenic adaptation which was prevented by training with superimposed vibrations. In other words, the presented data suggest that superimposing a vibrations stimulus to resistance exercise might not be beneficial for triggering angiogenic-inducing signaling pathways in skeletal muscle.

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4.3 Manuscript 1: 'The functional state of skeletal muscle circulation adapts differently to a resistive exercise intervention with and without superimposed whole-body vibrations.'

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Abstract

Whole-body vibration (WBV) training is today commonly practiced and previous research has suggested that it enhances peripheral circulation. Here we investigated muscle morphology and acute microcirculatory responses before and after a six-week training intervention applying resistive exercise (RE) and resistive vibration exercise (RVE) in 26 healthy males in a randomized, controlled parallel-design study. Total haemoglobin (tHb) and tissue oxygenation index (TOI) were measured in gastrocnemius muscle (GM) with near infrared spectroscopy (NIRS), whole-body oxygen consumption (VO_2) was measured via spirometry and skeletal muscle morphology was determined in soleus (SOL) muscle biopsies. Our data reveal that training-induced muscle deoxygenation was similar in both RE and RVE groups (P = 0.76), although VO_2 was higher in the RVE group. The RVE group showed an increased reactive hyperaemia in GM (P = 0.007) and increased blood volume (P < 0.01) after six weeks of training. The number of capillaries around fibres was increased after six weeks training in both groups (P < 0.001) with no specific effect of superimposed WBV (P = 0.61). The present findings suggest an increased blood volume and vasodilator response in gastrocnemius muscle as an adaptation to long-term WBV exposure combined with resistive exercise which was not observed after resistive exercise alone. We conclude that RVE-training has a specific effect on the vasodilator capacity of small arterioles and possibly capillaries. This effect might be advantageous for muscle thermoregulation and the delivery of oxygen and nutrients to exercising muscle.

INTRODUCTION

Whole-body vibration (WBV) was first applied in the training of athletes in 1985 and has received increasing scientific interest since [1]. Nowadays, WBV training is applied in various fields like sports, preventive medicine and rehabilitation [2,3]. Resistive exercise combined with WBV has been demonstrated to effectively attenuate disuse-induced muscle adaptations [4,5] such as reductions in fibre cross-sectional areas and capillary loss [6–8]. Consequently, resistive exercise combined with WBV is currently being considered as a potential training modality for future long-term human space missions [40].

While occupational exposure to high-frequency vibrations in the hand has been reported to induce vasospasm and tissue damage [9], brief exposures to low-frequency vibrations in healthy people acutely increases muscle perfusion and blood flow velocity during [10] and immediately after termination of WBV [11]. Furthermore, WBV is thought to increase blood viscosity within small vessels, resulting in increased total peripheral resistance followed by a compensatory dilation of arterioles [12]. Increased blood viscosity and elevated blood flow may both result in elevated shear stress in the microvasculature of skeletal muscle. This is significant as elevated shear stress can induce angiogenesis [13,14]. In support of this, it has been shown that WBV increases serum concentrations of pro-angiogenic vascular endothelial growth factor [15]. The angiogenic stimulus is potentially enhanced when combined with squatting, via eliciting muscle deoxygenation [16,17] which is also being considered as pro-angiogenic stimulation [18].

In the present study, we therefore hypothesized that superimposing whole-body vibration to resistive exercise would be a more potent pro-angiogenic stimulus than resistive exercise (RE) alone. More specifically, we hypothesized 1) that RE with superimposed WBV (RVE) depicts a more larger muscle deoxygenation and exercise-induced hyperaemia than RE alone and 2) that this effect would lead to more pronounced long-term adaptations, such as increased capillarity and blood volume in SOL after an exercise period of 6 weeks of RVE compared to RE. This study is to our knowledge the first to evaluate acute responses of WBV superimposed upon resistive exercise before and after a long-term training intervention with concurrent morphological analyses in muscle tissue. Our aim was to design a novel training approach that simultaneously stimulates muscle hypertrophy as well as capillarization to ensure optimal flux of oxygen and nutrients to the muscle in order to improve muscle performance.

MATERIAL AND METHODS

Study design

After comprehensive medical examinations, twenty-six recreationally active, healthy male subjects (age: 26 yrs (SD 4); height: 1.79 m (SD 0.04); body mass: 74.7 kg (SD 7.7); body mass index: 23.3 $kg \cdot m^{-2}$ (SD 1.96)) were included into this randomized controlled training study. All subjects provided written informed consent before participation in the study. The study was conducted in a two-group parallel design, in which one group performed resistive exercise (RE) and the other group resistive exercise with simultaneous whole-body vibrations (Resistive Vibration Exercise: RVE). The study was carried out in compliance with the *Declaration of Helsinki* following approval by the Ethics Committee of the Northern Rhine medical association (Ärztekammer Nordrhein in Düsseldorf, application no. 2010-174).

Training design

A detailed description of the study and training design has been published elsewhere [18]. In brief, a training session was composed of a warm-up (two sets with each 10 squats and 15 calf raises with a 15-kg barbell) followed by the resistive exercise that consisted of three sets of squats and calf raises with a loaded barbell, separated by a one-minute break. The rhythm of the squats and calf rises was guided by a metronome. The squats consisted of a 2-s eccentric and 2-s concentric phase, and the calf raises of a 1-s eccentric and a 1-s concentric phase. First and second sets were composed of each 8 squats (= 32 sec) and 12 calf raises (= 24 sec) and in the third set, squats and calf raises were performed until exhaustion. Training weights were determined four weeks prior to the study and set at 80% of the One-Repetition Maximum and accounted for 75.2kg (SD 6.5) in the RE group and for 81.5 kg (SD 7.7) in the RVE group with no difference between groups (P = 1.0). The subjects in the RVE group performed the resistive exercise protocol with simultaneous side-alternating whole-body vibrations with a 6-mm peak-to peak displacement (Galileo® Fitness, Novotech, Germany), whereas subjects in the RE group trained without superimposed vibrations.

Long-term study design

Participants concluded 16 exercise sessions in a period of 6 weeks (week 1-2: two training sessions per week; week 3-6: three training sessions per week). Training weights and vibration frequencies were progressively increased during the 6-week period. Vibration frequencies in the RVE group were increased from 20 Hz in the first week to 40 Hz during the

last two weeks with 5-Hz weekly increments. Training load was adjusted after each exercise session according to the subjects' individual training progressions applying the method described by Baechle and Earle [19] and using the number of repetitions performed in the third set of squats as a reference. So, if the subject completed eight repetitions in the third set, training load remained unchanged for the subsequent training session. If the participants conducted more or less than eight repetitions, training load was recalculated, i.e. increased or decreased, respectively, for the subsequent training session. The increase in training load over the 6-week intervention was 59.8 % (SD 17.3) in the RE group, which trained with 130.2 kg (SD 18.5) during the final exercise session. Training load was only increased by 46.9 % (SD 19.0) in the RVE group, which was significantly smaller compared to the RE group (time * intervention: $P < 0.001$). During the final training, RVE subjects trained with 110.2 kg (SD 15.8). Increase of training load in the RVE group was probably hampered by training with vibration frequencies above 35 Hz during the last two weeks, as discussed in the methodological paper previously published [20].

Near Infrared Spectroscopy

Near Infrared Spectroscopy (NIRS) utilizes differential absorption properties of oxygenated and deoxygenated haemoglobin and myoglobin at 760 and 840 nm [21–23]. NIRS is a useful technique to continuously evaluate tissue oxygenation index (TOI) and total haemoglobin (tHb) content in resting and exercising skeletal muscle [21]. The NIRS device used in this study was custom-built at the Rhein-Ahr Campus (University of Applied Sciences, Koblenz, Germany) and the mode of operation has been published elsewhere [24]. During the first and final training session of the 6-week intervention, NIRS data were collected at a frequency of 0.4 Hz. The NIRS optode was fixed above the lateral gastrocnemius muscle (GM) of the right leg and the distance to malleolus medialis and tibia edge was documented to ensure that the optodes were placed at the same location during the follow-up measurement. Prior to training, 5-min baseline data were collected while the subject was seated with a knee angle of 90°. Markers in the recorded files indicated baseline, warm-up, squats and calf raises. A typical data set is illustrated in Figure 1.

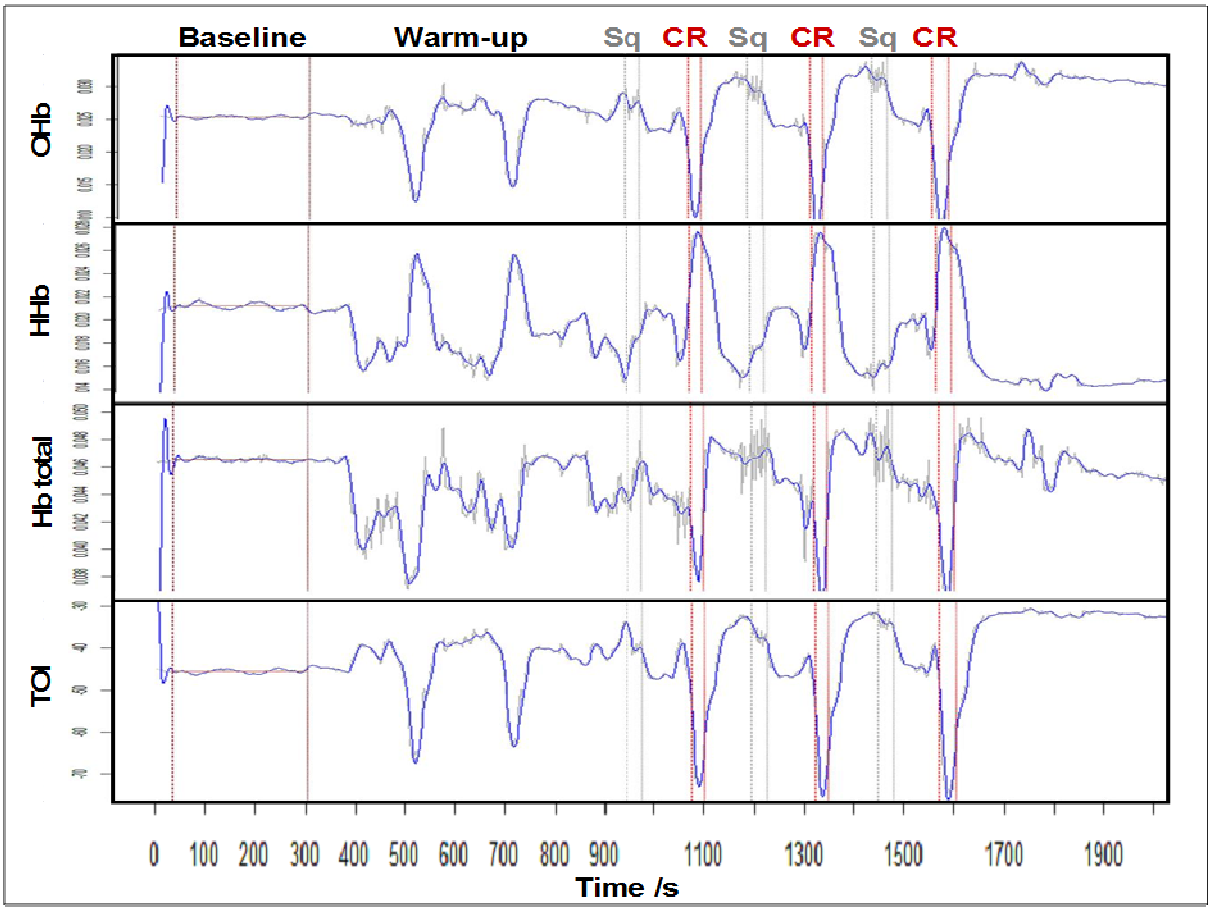

Figure 1. Illustration of NIRS data during training. Horizontal lines indicate marker positions. From left to right: first and second markers indicate baseline measurement, followed by a warm-up period. Following markers indicate starting and end points of squatting exercise (Sq) and calf raises (CR), respectively. OHb=oxygenated haemoglobin, HHb=deoxygenated haemoglobin, tHb=total haemoglobin, TOI=Tissue Oxygenation Index.

Data processing:

NIRS data were processed using R statistical computing software (version 2.14.2; www.r-project.org). A 4th-order Butterworth low pass filter (cut-off frequency = 0.08 Hz) was used for noise reduction. NIRS measures oxygenated (OHb) and deoxygenated (HHb) haemoglobin with myoglobin playing only a minor part (< 20%) [25]. Total haemoglobin (tHb) was calculated as OHb + HHb. The tissue oxygenation index (TOI) was computed as OHb / (OHb + HHb). We were particularly interested in changes in these parameters during and after calf raises, as this movement is expected to have the greatest impact on the oxygenation in the gastrocnemius muscle.

A custom-made R-script was used to extract the data (see Fig. 2). Means were calculated during baseline, generally excluding the initial 30 s of the data recordings. Furthermore, the minimum values of TOI during calf raises (Min_{during}) and the corresponding tHb values at the same time point were extracted as well as the maximum values of each TOI and tHb after

calf raises (Max_{Post}). From these values, relative changes between baseline and Min_{during} were calculated to evaluate muscle deoxygenation during calf raises (ΔTOI) as well as changes between baseline and Max_{Post} to determine reactive hyperaemia immediately after calf raises (ΔtHb).

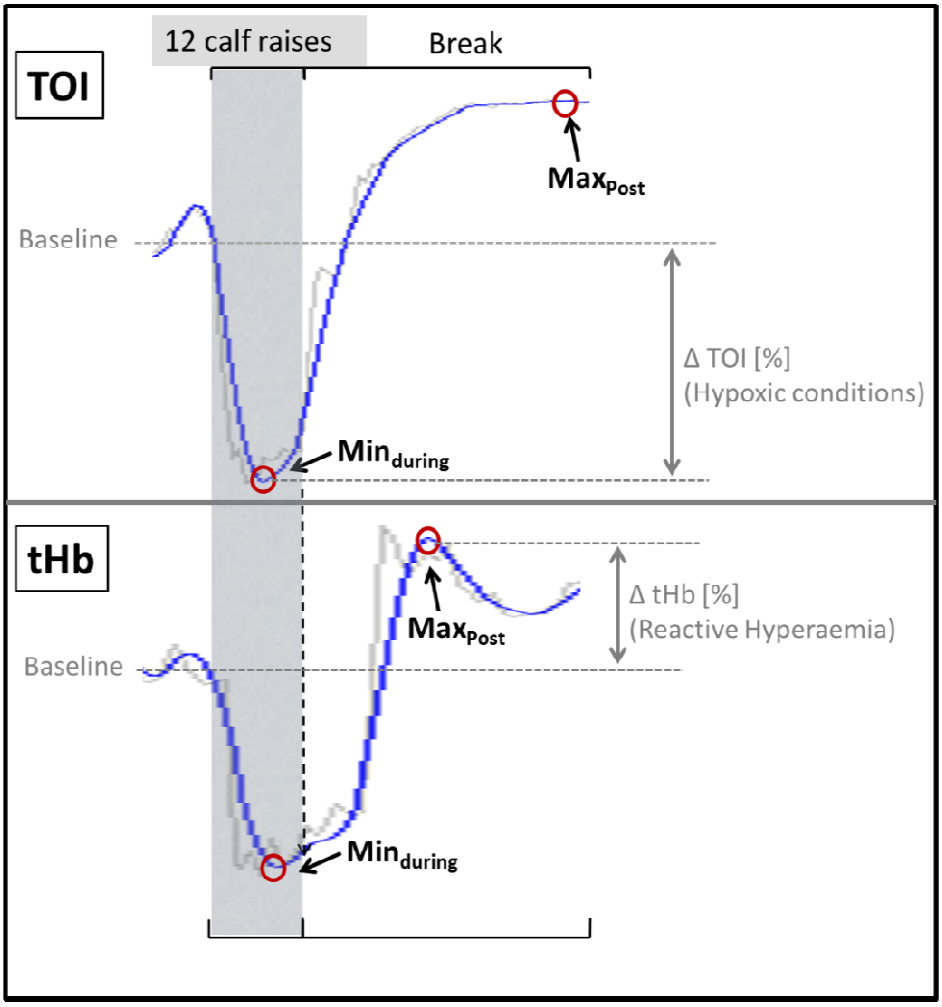

Figure 2. Illustration of the extracted data. Tissue oxygenation index (TOI) and total haemoglobin (tHb) during a single set of calf raises followed by a rest period in standing position ('Break') collected at a frequency of 0.4 Hz. Minimum values during a single set of calf raises followed by a rest period in standing position ('Break') collected at a frequency of 0.4 Hz. Minimum values during calf raises (Min_{during}) and maximum values during the subsequent break (Max_{Post}) were identified. ΔtHb: relative change in total haemoglobin from baseline; ΔTOI: relative change in Tissue Oxygenation Index from baseline

Acute study evaluating the effect of different vibration frequencies upon NIRS data

An acute training study including four healthy male subjects (age: 27.5 yrs (SD 7.0); height: 1.82 m (SD 0.05); body weight: 78.7 kg (SD 7.6), body mass index: 23.8 (SD 2.0)) was conducted in a cross-over design to evaluate the effect of two different vibration frequencies (20 Hz and 40 Hz) on GM deoxygenation during training and reactive hyperaemia after training. The two training sessions were separated by one week and the weights lifted were 85 ± 11 kg. The training conditions were identical to those applied in the long-term study with the only difference that the third set was composed of 8 squats and 12 calf raises. NIRS data were captured as described above.

Spirometry

Whole-body oxygen consumption (VO_2) during training was measured via spirometry with a portable spirometer (Oxycon^{TM} Mobile, CareFusion, Rolle, Switzerland). Measurements were performed at the initial and final exercise sessions of the 6-week training intervention. VO_2 data during calf raises were normalized to the subjects' body weight and are presented in [ml/kg$_{(BW)}$], where BW is body weight. For the present paper, mean values for the three sets were calculated in order to compare whole-body oxygen consumption with local oxygen consumption that were measured via NIRS.

Soleus muscle morphology

Collection of muscle biopsies

Soleus muscle (SOL) biopsies were collected one week before the initial exercise and 3 days after the final exercise session. Biopsies were taken after an overnight fast (≥ 8 hours) from the lateral side of the SOL muscle, approximately 1 cm below the belly of the lateral gastrocnemius muscle. The disinfected skin surface area was anesthetized with 1.5 to 2 ml of 2% lidocaine solution and a 10-mm incision through skin and muscle fascia was made. Muscle samples were taken with a Weil–Blakely rongeur (Gebrüder Zepf Medizintechnik, Tuttlingen, Germany) and snap frozen while agitating rapidly in liquid nitrogen and stored at -80°C for further analyses.

Histological Methods

Transverse 10-µm muscle sections were cut in a cryostat at -20°C, transferred to microscope slides, dried for 30 minutes and stored at -80°C until analysis. For histochemical analyses,

sections were thawed and dried for 10 minutes at room temperature, fixed for 10 min in pre-cooled acetone (-20°C), blocked for one hour in 5% Bovine Serum Albumin (BSA: # A9418**,** Sigma-Aldrich, St. Luis, US) dissolved in Tris Buffered Saline (TBS: TRIS-HCl [0.05 M], NaCl [0.6 M], pH 7.6). Then they were co-incubated over night with primary antibodies against Myosin Heavy Chain Type I (#A4840, Developmental Studies Hybridoma Bank, Iowa City, US) and Caveolin-1 (#SP5142P, Acris, San Diego, US). After incubation with appropriate biotinylated secondary antibody (DakoCytomation, Glostrup, Denmark) and Horseradish Peroxidase (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) for each hour each; the final reaction was carried out using 3,3`-diaminobenzidine (DAB) solution (phosphate buffer [0.09 M], pH 7.4, DAB [2.2 mM], ammonium chloride [7.03 mM], nickel sulphate [0.93 mM], ß-D-glucose [10.44 mM] and glucose oxidase [24 nM]). After dehydration, sections were embedded in Entellan (#107960, Merck, Darmstadt, Germany) and mounted with a cover slip.

Analysis of stained sections:

Stained biopsy sections were photographed with a USB-Monochrome camera with a 1280x960 pixel chip (ICX205AL, Sony Corporation, Tokyo, Japan) using a light microscope (Axio Scope.A1, Carl Zeiss Microscopy GmbH, Göttingen, Germany). Areas in the muscle section with perpendicularly cut fibres (i.e. circular or polygonal in shape) were chosen for analysis and areas with longitudinally or obliquely cut fibres were avoided. Images were analysed by the same operator using the custom-made 'Histometer' software (Version 1.3d), which was implemented as plugin into the ImageJ image processing software (ImageJ 1.46r, National Institute of Health, US). The following morphological parameters were calculated: capillary density (CD; capillaries·mm^{-2}) as the overall number of capillaries divided by the area of the region of interest (ROI); capillary-to-fibre ratio (C:F ratio) as the overall number of capillaries divided by the overall number of fibres; capillaries around fibres (CaF) as the number of capillaries surrounding a fibre (distance between capillary and fibre < 20 pixels ≙ 9.3 µm); fibre cross sectional areas (FCSA) [μm^2]; smallest fibre diameters (DiaMin) as the smallest diameter of each fibre crossing the centre of gravity [µm]; fibre type distribution as the relative distribution of type I and type II fibres. The average number of fibres analysed per section was 106 (SD 51), depending on size, shape and quality of the section.

Statistical Analyses

Statistical analyses were performed using STATISTICA 10 for Windows (Statsoft, Tulsa, Oklahoma, USA, 1984-2010). For NIRS and morphological data, a repeated-measures ANOVA was applied with time as within-subject factor (pre *vs.* post exercise) and intervention as between-subject factor (resistive exercise *vs.* resistive vibration exercise). Tukey's test was used for post-hoc testing. For evaluation of the effect of different vibration frequencies in the acute study, a two-sided, paired Student's T-test was performed. Values are given as means ± SD; delta values are given in per cent change from baseline. Statistical significance was set at $P < 0.05$.

RESULTS

Levels of tHb and TOI in GM were comparable between the three sets of calf raises at Min_{during} (tHb: $P = 0.42$; TOI: $P = 0.52$) and at Max_{Post} (tHb: $P = 0.14$; TOI: $P = 0.50$). Therefore, values of the three sets per training were lumped together as means and used for further analysis.

Angiogenic stimuli during exercise

Deoxygenation during training

TOI, a marker of muscle oxygenation, decreased in GM during calf raises by -35.0 % (SD 24.5) in the RE group and by -37.2 % (SD 26.7) in the RVE group during the initial training. The training response did not differ significantly between RVE and RE groups (initial training: $P = 0.76$; final training: $P = 0.31$) nor was there a detectable long-term adaptation of the acute responses to the 6-week training intervention (RE: $P = 0.89$; RVE: $P = 0.23$), see Figure 3A.

Reactive Hyperaemia as an indicator of increased shear stress

An increase in tHb over baseline is indicative for reactive hyperaemia. There was an overall effect of group, which suggested that the RVE group exhibited a larger reactive hyperaemia than the RE group ($P = 0.007$). This difference seems to be induced by increased reactive hyperaemia in the RVE group after the 6-week intervention, reflected by a trend in the group*time interaction ($P = 0.094$), see Figure 3B.

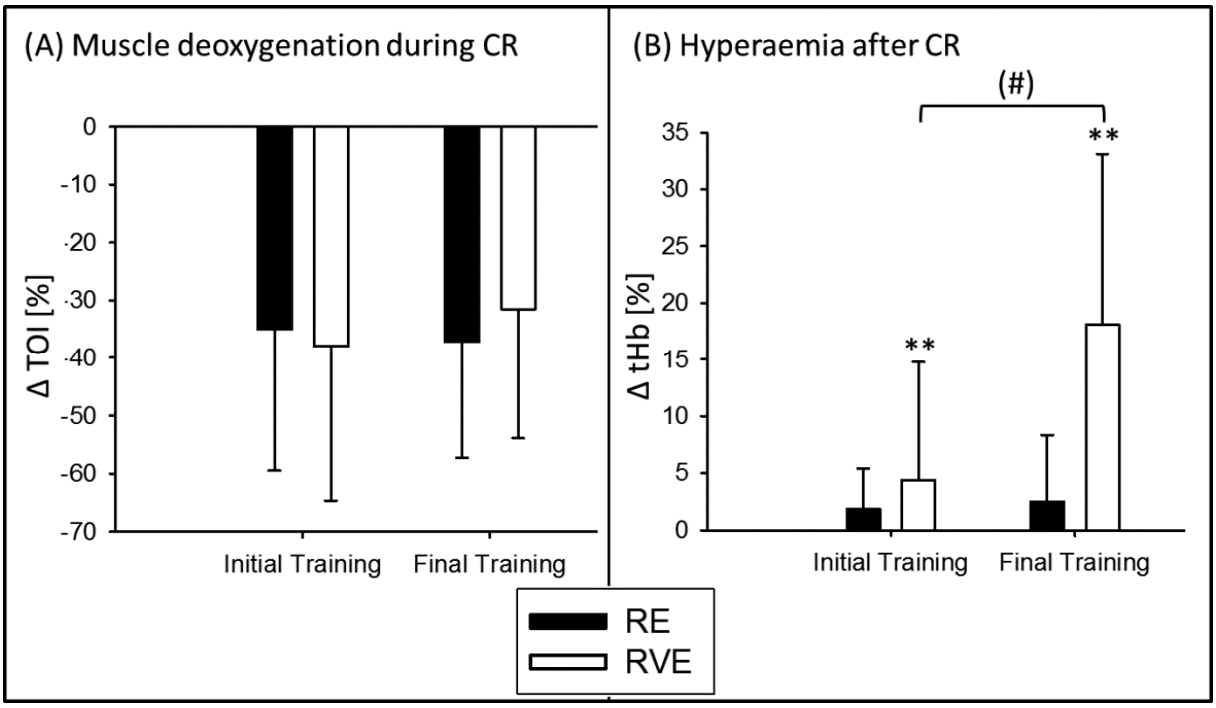

Figure 3. Evaluation of deoxygenation and reactive hyperaemia. RE: resistive exercise, RVE: resistive vibration exercise, n = 13. **(A)** Deoxygenation during calf raises (ΔTOI: tissue oxygenation index) was comparable between the two groups (P = 0.79) and training types (*P* = 0.5). (**B)** reactive hyperaemia after calf raises (ΔtHb: total Haemoglobin) were assessed as relative changes from baseline. The RVE group depicted higher hyperaemia compared to the RE group (** group effect: *P* = 0.007) with a trend towards an enhanced hyperaemic response after 6 weeks of RVE (time*intervention effect: $^{(\#)}P$ = 0.094).

The effect of different vibration frequencies on hypoxia and reactive hyperaemia

As the subjects trained with 20 Hz vibration during the initial training and with 40 Hz vibration during the final training, the results in Figure 3 do not allow discrimination between the effect of different vibration frequencies and long-term training adaptations. To rule out the bias induced by training at different frequencies, we conducted a post-hoc acute study including four subjects to test the impact of these different frequencies on TOI and tHb. We observed that no significant difference in GM deoxygenation (*P* = 0.31) or reactive hyperaemia (*P* = 0.36) during 20-Hz and 40-Hz calf raises, see Figure 4.

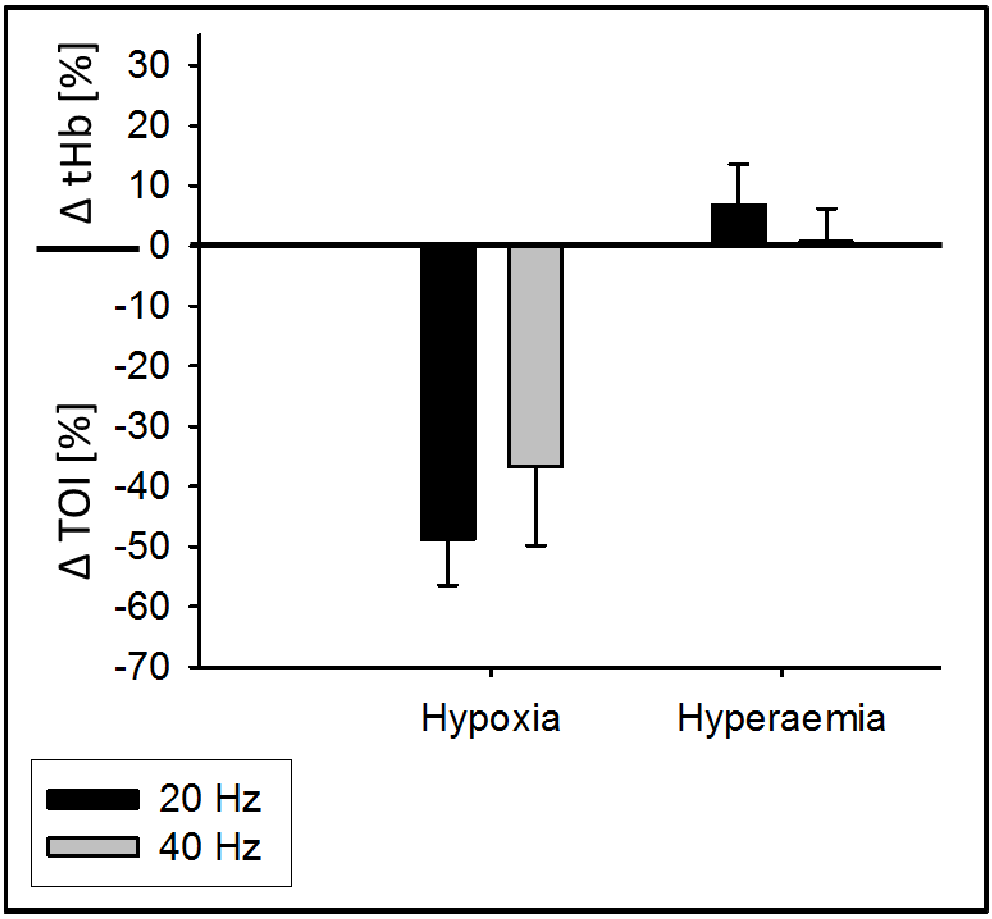

Figure 4. The effect of different vibration frequencies upon deoygenation and reactive hyperaemia during resistive vibration exercise. Deoxygenation during calf raises (ΔTOI: tissue oxygenation index) and reactive hyperaemia after calf raises (ΔtHb: total Haemoglobin) were assessed as relative changes from baseline, n = 4. There was no significant differences in responses when training at 20 Hz compared to 40 Hz. Moreover, the non-significant differences between frequencies are opposite to the changes observed between pre and post in the main study, underpinning the notion of adaptation to training.

Adaptations to the 6-week training intervention

Total Haemoglobin

Absolute tHb values were measured in GM pre and post the 6-week training intervention. We found that the tHb in GM was higher at all measured time points (Baseline: $P = 0.004$, Min_{during}: $P < 0.001$; Max_{Post}: $P < 0.001$) after 6 weeks of RVE, but not RE (Baseline: $P = 0.59$; Min_{during}: $P = 0.98$; Max_{Post}: $P = 0.822$) compared to the initial training, see Figure 5.

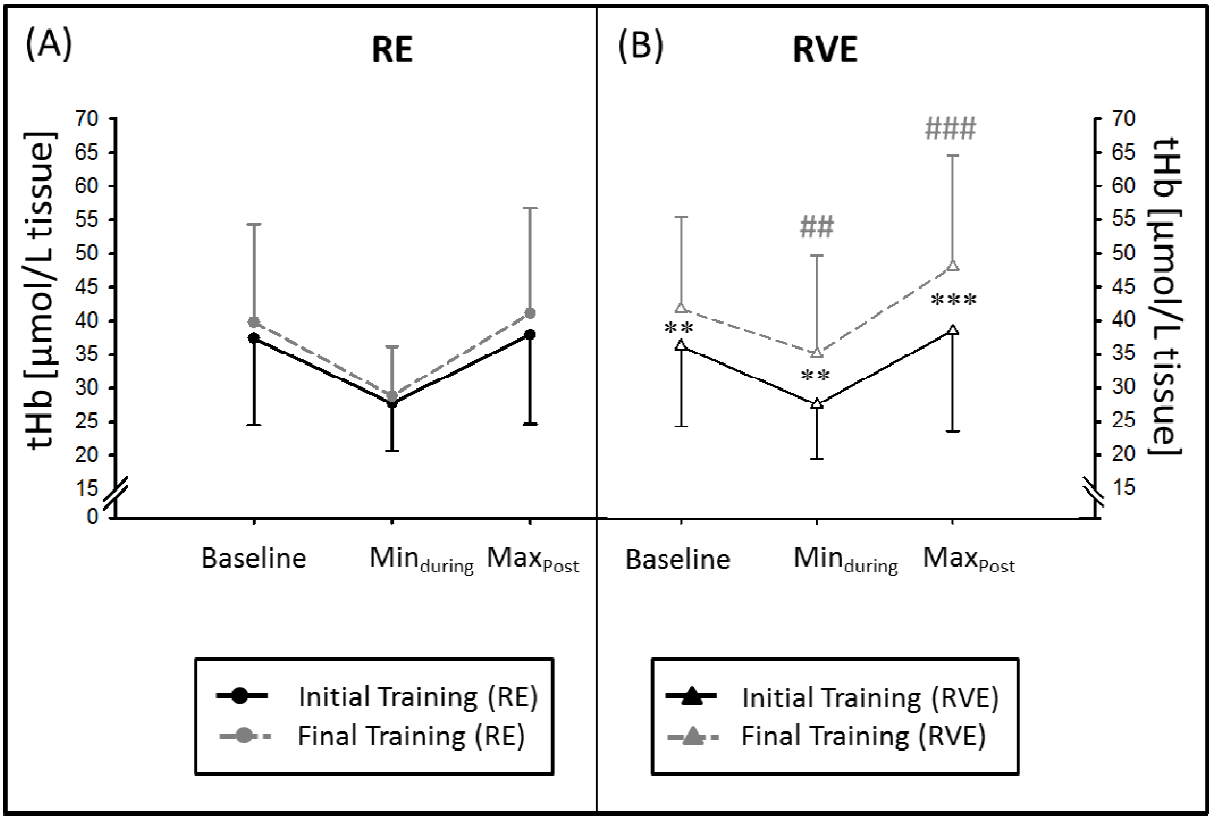

Figure 5: Total haemoglobin (tHb) content in gastrocnemius muscle at baseline, during and after calf raises. Data points represent means ± SD measured at the initial training (black) and final training (grey) of a 6-week training intervention with **(A)** resistive exercise (RE) or **(B)** resistive vibration exercise (RVE), n = 13. Min_{during}: minimum tHb concentrations during calf raises, Max_{Post}: maximum tHb concentrations acutely after calf raises. The RVE group depicted higher tHb levels after 6 weeks of training compared to the initial training: $^{**}P < 0.01$; $^{***}P < 0.001$. Differences between the two intervention groups during final training: $^{\#\#}P < 0.05$; $^{\#\#\#}P < 0.01$.

Tissue Oxygenation Index

The RE and RVE groups responded comparably to the trainings as there was no significant difference between groups ($P > 0.43$) in GM. Although the RE group showed decreased TOI during the final training compared to the initial training ($P = 0.024$), this effect has to be treated with caution as the group*time interaction revealed no difference between groups ($P = 0.33$), see Figure 6.

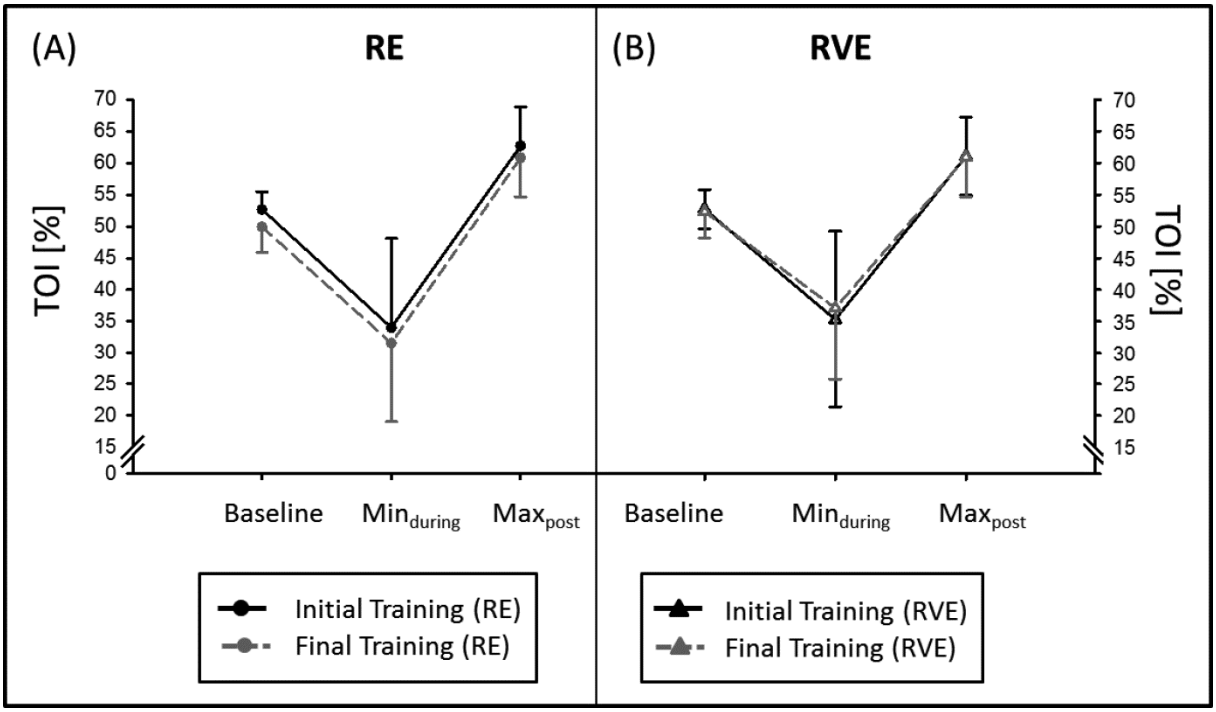

Figure 6: Tissue Oxygenation Index (TOI) in gastrocnemius muscle during and after calf raises. Data points represent mean TOI values (±SD) measured at the initial training (black) and final training (grey) of a 6-week training intervention with **(A)** resistive exercise (RE) or **(B)** resistive vibration exercise (RVE), n = 13. Min_{during}: minimum values during calf raises, Max_{Post}: maximum values acutely after calf raises. TOI levels were unaffected by the 6-week training intervention ($P > 0.43$)

Whole-body oxygen consumption during calf raises

The RVE group had a higher whole-body oxygen consumption (VO_2) during calf raises compared to the RE group, as represented by a significant group effect ($P < 0.001$). Furthermore, there was a long-term adaptation to the 6-week training intervention and VO_2 was higher in both groups during the final training compared to the initial training (intervention effect: $P = 0.0018$) with similar adaptations in both groups (time*intervention effect: $P = 0.69$), see table 1.

	VO_2 [ml/kg $_{(BW)}$] Initial Training	VO_2 [ml/kg $_{(BW)}$] Final Training
RE	18.4 ± 6.5	$21.2^{\#}$ ± 3.0
RVE	22.0^{*} ± 3.5	$25.5^{*\#}$ ± 2.6

Table 1. Whole-body oxygen consumption during calf raises. Values represent consumed volume of oxygen (VO_2) per body mass ($kg_{(BW);}$ BW: body weight) and are calculated as means of three sets of calf raises. The RVE group had a higher whole-body oxygen consumption compared to the RE group (group effect: $^{*}P < 0.001$). Both groups showed increased VO_2 after the 6-week training intervention (intervention effect: $^{\#}P = 0.0018$) with no significant time*intervention effect ($P = 0.69$).

Capillarity and morphology in SOL pre and post the 6-week training intervention

After both 6 weeks of RE and RVE, the mean number of capillaries around a fibre was significantly increased ($P < 0.001$) in SOL. The absence of a significant intervention * time interaction ($P = 0.27$) indicates that this response was similar in both groups. The capillary-to fibre ratio and capillary density did not differ significantly between groups or before and after training (Table 2).

Group	RE	RVE
CaF pre	4.77 ± 1.30	4.91 ± 1.26
CaF post	5.66 ± 1.30	5.44 ± 0.79
P- values	*Time effect: P < 0.001; Group effect: P = 0.89; Interaction effect: P = 0.27*	
CD pre	309.2 ± 87.7	297.2 ± 71.9
CD post	357.3 ± 12.9	337.8 ± 73.1
P- values	*Time effect: P = 0.081; Group effect: P = 0.61; Interaction effect: P = 0.87*	
C:F ratio pre	3.76 ± 0.89	5.10 ± 2.79
C:F ratio post	4.04 ± 0.95	4.080 ± 0.85
P- values	*Time effect: P = 0.39; Group effect: P = 0.77; Interaction effect: P = 0.82*	

Table 2. Capillarity in soleus muscle pre- and post the 6-week training intervention. CaF: capillaries around fibres; CD: capillary density; C:F ratio: capillary-to fibre-ratio; RE: resistive exercise, RVE: resistive vibration exercise. The number of capillaries around fibres was significantly increased by the 6-week training interventions.

The size of the fibres in SOL was similar in both groups and neither size nor fibre type composition were significantly changed after either intervention (Table 3).

Group	**RE**		**RVE**	
Muscle fibre Type	**Type I**	**Type II**	**Type I**	**Type II**
FCSA pre [µm^2]	10187 ± 3291	16102 ± 8230	9920 ± 2977	11865 ± 3530
FCSA post [µm^2]	11278 ± 62211	17271 ± 10039	9804 ± 3084	12965 ± 3699
P-values (pre vs. post)	*0.93*	*0.97*	*0.99*	*0.99*
DiaMin pre [µm]	56.77 ± 8.39	69.67 16.04	62.56 ± 8.26	67.88 10.31
DiaMin post [µm]	57.47 ± 8.36	72.83 16.71	60.71 ± 6.21	69.54 7.55
P-values (pre vs. post)	*0.99*	*0.91*	*0.53*	*0.99*
Fibre type distribution pre [%]	75.35 ± 12.81	24.65 ± 12.81	69.11 ± 16.54	30.98 ± 16.54
Fibre type distribution post [%]	74.52 ± 14.38	25.48 ± 14.38	71.24 ± 15.68	28.76 ± 15.68
P- values (pre vs. post)	*0.99*	*0.99*	*0.99*	*0.99*

Table 3. Morphological data in soleus muscle pre and post the 6-week training intervention. FCSA: fibre cross-sectional area, DiaMin: minimum fibre diameter RE: resistive exercise, RVE: resistive vibration exercise. Morphological parameters were not changed by the 6-week training intervention.

DISCUSSION

The main observation in the present study is that a 6-week training program with whole-body vibrations superimposed on resistance exercise (RVE) results in a larger reactive hyperaemia and increased blood volume in GM compared to 6 weeks of resistance exercise (RE) only. Despite these findings, muscle oxygenation is similar in both conditions. While this could imply a better perfusion of the RVE- than RE-trained muscles, the capillary bed in SOL was not differentially affected by the two training programs. Thus, despite the higher reactive hyperaemia, and hence albeit increased mechanical stress acting on endothelial cells during RVE, the vibration training regime was not sufficient to augment structural adaptations in terms of capillary formation in SOL.

RVE does not aggravate muscle deoxygenation compared to RE

Previous studies show that WBV moderately increases muscle activity [29–31] and ATP consumption [32]. In line with this it has been reported that WBV can induce muscle deoxygenation and serve as a training stimulus [33,34]. Based on these reports we hypothesized that WBV superimposed on resistance exercise would result in a more pronounced deoxygenation of the muscle than resistance exercise only. In contrast to our expectation, we observed that gastrocnemius oxygenation was decreased similarly by short-duration exercise (~ 24 s) performed with heavy loads with or without WBV. This finding is in line with previous studies, which likewise showed no vibration specific effect upon tissue oxygenation in vastus lateralis and GM muscles during static squats [35] or during isometric contractions of the calf muscle [36]. The reason for the differential effects of WBV may be the training settings, which differed between studies: Mieva et al. [37] and Yamada et al. [38] applied lower vibration frequencies (10 and 15 Hz, respectively) and measured in vastus lateralis muscle. Furthermore, the two studies applied different training modes and durations of WBV exposure. Thus, WBV stimulation may have differential effects according to the way it is applied. Taken together, the available evidence suggests that superimposing WBV on resistance exercise does not enhance deoxygenation induced training adaptations.

We found that whole-body oxygen consumption (VO_2) was 16 % higher in the RVE than in the RE group during the final training (Table 1), even though the RE-trained subjects were able to lift significantly larger weights than the RVE group. It is not immediately clear how this can be explained, but it does suggest that the metabolic work at the end of the

intervention is larger in the RVE than RE group despite the lower training loads. From these data, one would expect the RVE group to have more pronounced muscle deoxygenation after the 6-week intervention than the RE groups. This was, however, not the case; the decrease in TOI was similar in both groups during calf raises. An accentuated decrease in TOI is most likely prevented by a concomitant improvement in muscle perfusion as reflected by the higher tHb after 6 weeks RVE, during calf raises that was not observed for the RE group.

Six-week RVE intervention increases blood volume and dilation capacity of arteries in GM

The increased reactive hyperaemia after training and the increased blood volume at baseline after 6 weeks RVE may be due to capillary proliferation. At first glance the increase in the mean number of capillaries around SOL fibres supports this notion, but there are two problems with this interpretation. First, similar increases were seen after 6 weeks of RE and RVE, and probably more important is the observation that the capillary density was not significantly changed. Under the assumption that adaptations were similar in SOL and GM muscles (assessed by both biopsy and NIRS in this study), the augmented tHb levels in the RVE group are thus probably not associated with increased capillarity but could be derived from an increased number of perfused capillaries, which hints to a functional adaptation. The perfusion of the capillary bed controlled by arterioles [39] and the measured increase in tHb might thus derive from increased vasodilation of feeding arteries and arterioles. An increased perfusion would mean an increased flow through the feeding artery. Weber and colleagues (2012) demonstrated, however, in the participants of the present study that while 6 weeks of RE and RVE increased the diameter of the superficial femoral artery (SFA), there was no additional effect of superimposed WBV and flow-mediated dilation (FMD) was unaltered by the training intervention [40]. An explanation for this conundrum could be that WBV might lead to increased vasodilator capacity of small arterioles downstream of the SFA. This assumption is supported by Yamada and colleagues, who linked the augmented blood flow induced by WBV to an increased dilation capacity of small arterioles, while dilation capacity of conduit arteries was unaltered [41]. Thus, our data might reflect a redistribution of the blood flow due to an improved vasodilator capacity in resistance arteries of the lower leg, but not the thigh (SFA). A possible explanation might be that accelerations during WBV decrease with increasing distance from the vibration platform due to damping properties of the muscles and tendons [42]. WBV has been reported to increase intraluminal shear stress in blood vessels [2,43]. Various studies have shown that shear stress affects endothelial

nitric oxide synthase (eNOS) mRNA and protein expression [44–47] and shear stress furthermore activates NOS in red blood cells (RBC) [48]. It is therefore tempting to speculate that the observed increases in tHb in the present study derived from WBV-induced increases in shear stress, which subsequently increased expression and activation of eNOS and potentially RBC-NOS, thereby increasing NO-mediated vasodilation. Another possible explanation might thus be structural adaptation in capillary diameters i.e. that the capillary radius was increased after training intervention upon increased NO production as it has been observed in rat soleus muscles that the luminal diameter of capillaries is reduced after hindlimb suspension (Kano et al. 2000). Another potential explanation for the increased blood volume in GM after 6 weeks of RVE might be related to the so-called 'tonic vibration reflex', which elicits a high frequency of action potentials at neuromuscular endplates upon vibration exposure [49]. This might increase acetyl choline spillover at neuromuscular endplates, which is known to be a potent vasodilator [50]. Furthermore, with each action potential, potassium is released from skeletal muscle, which also has vasodilating properties [51]. However, these interpretations are hypothetical and remain to be clarified in future studies.

It is interesting to note that resting tHb and reactive hyperaemia after training were higher after six weeks of RVE than RE. Yet, TOI and deoxygenation was the same in both conditions. Thus, our data indicate that adaptations induced by 6 weeks of RVE increase oxygen demand of the tissue. As discussed above, WBV-induced dilation may over time change the functional status, but not the morphology of the microvasculature, where there is a redistribution of blood to the vibrated muscles. One possible explanation could be that this increased flow and commensurate increase in oxygen extraction even during rest (as the TOI is the same) is due to repair of tissue damage induced by vibration. However, there is no evidence that WBV superimposed on resistance exercise does cause damage [52]. It remains unknown what causes this increased oxygen demand and flow, even at rest, after 6 weeks RVE. One explanation could be a higher basal tone of the muscle.

SOL morphology

Our morphological data in SOL indicate that six weeks of RE or RVE did not induce any fibre type shift or hypertrophy. The absence of hypertrophy in SOL has been confirmed via magnetic resonance imaging measuring muscle volume pre and post training intervention within the same study (unpublished observations).

Study limitations

Morphological and functional data in the present study were captured in different muscles (SOL and GM, respectively). The underlying reason was that preliminary testing revealed that NIRS measurements in SOL muscle during WBV were not feasible and therefore data were captured in GM. Biopsies were however only available from SOL, which had been considered for other measurements in the study protocol and a post-hoc decision was made to determine SOL morphology. However, we expect that an intervention-specific adaptation would not be limited to certain muscles and we therefore assume that the increases in SOL capillarity, which we observed after 6 weeks of training with no group-specific effect, can be transferred from SOL to GM muscle.

Summary and Conclusion

In summary, our data indicate that a whole-body vibration stimulus superimposed to resistive exercise does not accentuate muscle deoxygenation during a training session but at the same time increases whole-body oxygen consumption. Also morphological adaptations in the microvasculature after six weeks resistance exercise were similar after 6 weeks of resistance exercise with or without whole body vibrations. Yet, total blood content in the gastrocnemius muscle was specifically enhanced in the group that performed resistive exercise with superimposed WBV, and so was reactive hyperaemia. We therefore conclude that RVE-training has a specific effect on the functional state of small arterioles and possibly capillaries and that a potential explanation for this could imply shear-stress induced chronic increases in NO-mediated vasodilation of small arterioles.

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5 Primary Findings of the Thesis and Conclusion

We could show in paper 1 that resistance exercise combined with superimposed whole-body vibrations (WBV) at frequencies between 20 and 30Hz was well tolerated. However, training with vibration stimuli of 40Hz hampered the increase of training load compared to the group that trained without WBV and additionally, seven out of thirteen subjects complained about low back pain when performing resistance exercise with 35-40Hz vibration. These results allow the recommendation that side-alternating WBV above 35Hz should not be applied in combination with heavy training loads, as it might reduce training performance and foster back pain. Furthermore, resting systolic and diastolic blood pressures were decreased in both groups after the six-week resistance training intervention. It is commonly accepted that endurance exercise has beneficial effects upon cardiovascular health [1,2]. Conversely, studies on cardiovascular adjustments to long-term resistance exercise remain inconclusive. Previous studies show increased [3] or decreased [4] resting blood pressure in resistance-trained athletes and resistance exercise has also shown to be effective to decrease blood pressure in obese and hypertensive subjects [5–7]. However, limited data are available in healthy recreationally active people and our findings reveal that resistance exercise potentially exerts a beneficial effect to the heart.

To test the hypothesis that the superposition of WBVs to resistance exercise adds a pro-angiogenic stimulus to the training, we evaluated serum concentrations of the angiogenic markers MMP-2, MMP-9, VEGF and endostatin in exercise-trained human subjects and determined their proliferative capacity upon endothelial cells *in vitro* (paper 2). The data show that both RE and RVE induced transient increases in circulating pro-angiogenic markers which all depicted maximum concentrations two minutes after exercise. This is a novel finding as previous studies evaluated the effect of endurance exercise on these markers [8–14]. According to our hypothesis, we would have expected higher post-exercise VEGF concentrations in the RVE group. Contrarily, our data reveal that the superposition of WBV to resistance exercise decreases circulating post-exercise VEGF concentrations and this effect supposedly results in reduced EC proliferation *in vitro*. Post-exercise endostatin concentrations were elevated after six weeks of RE, which according to Schmidt and colleagues (2004) can be considered a pro-angiogenic training adaptation as endostatin concentrations that we measured (ranging from 90-140 ng/mL) lie close to concentrations

that were shown to induce EC proliferation and migration [15]. This training adaptation was inhibited by training with superimposed WBV.

From the VEGF and endostatin data in paper 2, one would suggest that superimposing WBV to RE might not be beneficial for promoting angiogenesis in skeletal muscle. The data in manuscript 1, however, do not confirm this conclusion, as capillarity in soleus muscle did not adapt differently to the six-week training interventions RE and RVE. Hence, higher VEGF concentrations in serum after RE may be related to a systemic training effect, e.g. increased endothelial cell regeneration rather than reflecting angiogenic events occurring in muscle tissue. As the serum was taken pre and post training, another possible explanation might be that VEGF concentrations were comparable or even higher in the RVE during training and that the time curve might have been shifted to the left by WBV stimulation.

Another interesting finding in paper 2 was that resting and post-exercise MMP-2 levels were increased by the six-week RVE intervention. As MMP's have multiple functions in the body, this finding does not necessarily reflect increased angiogenic stimulation in muscle tissue but might for example indicate increased IGF-associated anabolic stimulation upon long-term WBV exposure, as MMP-2 has been shown to increase IGF bioavailability via degradation of the IGF binding protein [16,17].

The main observation in Manuscript 1 is that a six-week training program with WBV superimposed on resistance exercise results in a larger hyperaemic response and increased blood volume in gastrocnemius muscle compared to six weeks of resistance exercise only. Despite these findings, muscle oxygenation is similar in both conditions. While this could imply a better perfusion of the RVE- than RE-trained muscles, the structure of the capillary bed was not differentially affected by the two training programs. These data indicate that regular exposure to WBV in combination with resistance exercise influences the functional state of small arterioles and potentially capillaries, possibly via shear-stress induced chronic increases in NO- mediated vasodilation. Hence, WBV-induced dilation may over time change the functional status, but not the morphology of the microvasculature.

We conclude that the superposition of WBV to RE acutely decreases circulating concentrations of angiogenic factors (VEGF and endostatin), which supposedly does not influence capillary growth in skeletal muscle as we saw similar adaptations in skeletal muscle

morphology in both groups after six weeks of training. Functional measurements indicate that six weeks of RE training with superimposed WBV increases skeletal muscle perfusion. Hence, WBV may influence the functional state of small arterioles and potentially capillaries but does not induce additional capillary growth. The mechanisms leading to increased muscle perfusion remain unknown and represent an interesting target for future investigations.

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6 Appendix

6.1 Abstract (German) / Zusammenfassung

Hintergrund: Ganzkörper-Vibrationstraining (‚Whole-Body Vibration', WBV) ist in den letzten Jahren zu einer beliebten Trainingsmethode geworden und findet heutzutage in vielen Bereichen Anwendung, z.B. in Fitnessstudios oder in der Rehabilitations-und Präventionsmedizin. Bisherige Studien zeigen, dass WBV u.a. einen positiven Effekt auf die Durchblutung hat und eine Deoxygenierung der vibrierten Muskeln herbeiführen kann. Die dieser Arbeit zu Grunde liegende Hypothese ist, dass Ganzkörpervibrationen in Kombination mit konventionellem Widerstandstraining einen zusätzlichen angiogenen Stimulus erzeugen. Ziel war es, eine neue Trainingsmethode zu entwickeln und wissenschaftlich zu überprüfen, die gleichzeitig Hypertrophie und Kapillarwachstum im Muskel induziert und dadurch Muskelleistung im Sinne von Maximalkraft und Ermüdungswiderstandsfähigkeit verbessert.

Methoden: Eine sechswöchige Trainingsstudie mit 26 gesunden Männern wurde im Parallel-Design durchgeführt. Eine Gruppe trainierte mit konventionellem Widerstandstraining (‚resistive exercise': *RE*), während die andere Gruppe ein Widerstandstraining kombiniert mit Ganzkörpervibrationen (‚resistive vibration exercise': *RVE*) absolvierte. Die Probanden trainierten 2-3 Mal pro Woche. Das Training bestand aus Kniebeugen und Zehenständen, welche mit hohen Zusatzgewichten durchgeführt wurden (80% des *One-Repetition Maximums*). Funktionelle Messungen wurden während des ersten und letzten Trainings der sechswöchigen Intervention durchgeführt: jeweils vor, während und direkt nach dem Training. Muskeldurchblutung und Oxygenierung im Gastrocnemius Muskel wurden mit Nah-Infrarot Spektroskopie gemessen. Desweiteren wurden die Angiogenesemarker Matrix Metalloproteinase -2 and -9, Vascular Endothelial Growth Factor (VEGF) und Endostatin im Serum via ELISA gemessen und deren Effekt auf Endothelzellen (*human umbilical vein endothelial cells*) wurde *in vitro* bestimmt. Außerdem wurden Langzeit-Anpassungseffekte auf die Morphologie des Soleus Muskels bestimmt.

Ergebnisse: Nach dem Widerstandstraining konnten wir eine Erhöhung der gemessenen Angiogenesemarker im Serum feststellen. VEGF-Konzentrationen und Endothelzellen-Proliferation waren in der RE Gruppe höher im Vergleich zur der RVE Gruppe. Außerdem wurde nach der 6-wöchigen Trainingsintervention in der RE Gruppe eine erhöhte Endostatin-Konzentration direkt nach dem letzten Training gemessen, während solch ein Effekt in der

RVE Gruppe ausblieb. Morphologische Daten zeigen, dass die strukturellen Muskelanpassungen zwischen beiden Gruppen vergleichbar waren, obwohl sich die funktionelle Muskeldurchblutung der RVE Gruppe erhöhte.

Schlussfolgerungen: Die Daten zeigen, dass der Zusatz von Ganzkörpervibrationen zu konventionellem Widerstandstraining den pro-angiogenen Stimulus des Trainings nicht erhöht. Es wurde gezeigt, dass zwischen den beiden Gruppen strukturelle Muskelanpassungen vergleichbar sind. Dennoch scheinen Ganzkörpervibration, wenn sie in Kombination mit Widerstandstraining appliziert werden, die Dilatationsfähigkeit von Arteriolen und eventuell auch von Kapillaren zu beeinflussen was sich in der erhöhten funktionellen Muskeldurchblutung widerspiegelt.

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Original Article

Hylonome

Randomized controlled study on resistive vibration exercise (EVE Study): protocol, implementation and feasibility

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Abstract

Objectives: A training intervention comparing resistance exercise with or without whole-body vibration (WBV) was conducted to compare acute and chronic effects on functional and molecular parameters. **Methods:** A six-week training intervention was performed including 26 healthy males (26 years, SD=4). Two groups were analyzed in a parallel design performing either resistive exercise (RE, n=13) or resistive vibration exercise (RVE, n=13) training with weekly increasing vibration frequencies (20-40Hz). Resting and exercising blood pressure and heart rate were measured before and after the 6-week intervention. **Results:** Both training interventions decreased resting systolic blood pressure (P=0.003). Resting diastolic blood pressure was significantly decreased only in the RVE group (P=0.01). Exercising diastolic blood pressure was significantly decreased during the final training (P<0.001) with no additional effect of superimposed vibrations. Resistance exercise with superimposed vibrations evoked back pain to a higher degree than resistance exercise alone when training at frequencies above 30Hz (P<0.01). **Conclusions:** These data suggest positive effects of resistance exercise upon cardiovascular health and vascular responsiveness and a further beneficial effect of superimposed vibrations in decreasing resting diastolic blood pressure. Finally, development of back pain may be fostered by superimposed vibrations to high training loads, particularly at higher frequencies.

Keywords: Resistive Vibration Exercise, WBV, Blood Pressure, One-Repetition, Maximum, Training

Introduction

Regular performance of aerobic exercise is commonly known to have beneficial effects upon cardiovascular health such as decreases in heart rate and ambulatory blood pressure[1,2]. However, studies on cardiovascular adaptations to resistance exercise remain inconclusive. In the early 1980's, resistance exercise was believed to cause hypertension[3]. However, other studies showed that resting blood pressure was decreased by a resistance training intervention[4,5] whereas other studies showed no effect upon resting blood pressure in normotensive individuals[6-8]. The divergence in the reported effects indicates the need for further investigations in the field of cardiovascular adaptations to resistance exercise. Here we report acute and long-term responses of blood pressure and heart rate to a resistance training intervention performed with and without superimposed vibrations. Whole-body vibration (WBV) training has become increasingly popular during the past two decades and is nowadays applied in various fields like sport, rehabilitation and in clinical settings. Previous studies have made a great effort to describe physiological effects of whole-body vibration and have been reviewed elsewhere[9,10]. Unfortunately, many of the reported vibration-induced effects vary from study to study, which may derive from discrepancies in the applied training protocols, subject heterogeneity and divergence in the duration of the interventions. Furthermore, training supervision and diet control were neglected in many of the studies, and there was likewise no uniformity in the control conditions: studies either lacked a control group or compared their results to a passive

The authors have no conflict of interest.

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control group; only few studies applied an exercise control condition[10]. Also, there is a lack of consistency in the way of reporting the results, as highlighted in the recommendations of the international society of musculoskeletal and neuronal interactions[11]. Many of the potential benefits of whole-body vibration may thus not have been clearly demonstrated. To the best of our knowledge, no study has yet compared acute effects of a specific exercise to its long-term adaptations. However, and considering that exercise is usually conducted regularly and over a longer period of time, it is pertinent to ask whether long-term training alters acute responses and if superimposed vibrations promote a beneficial training effect. Here we present the design, feasibility and demands of a conducted study that allows investigation of the adaptation of acute responses during exercise to a long-term training intervention. Acute functional parameters (cardiovascular responses, neuromuscular activation, oxygen consumption, muscle perfusion and oxygenation) are complemented with investigations of acute responses on circulating factors in serum as well as acute and long-term responses within muscle tissue. The various measurements within a single training study using an exercising control group will hopefully provide a broader insight into the effects of the vibration stimulus *per se*. The present article focuses on acute and long-term cardiovascular responses as well as feasibility and demands of the training.

Material and methods

Study design

The EVE study ("Molecular and functional **E**ffects of resistive **V**ibration **E**xercise") was conducted in a two-group parallel design and was carried out in compliance with the *Declaration of Helsinki* following approval by the Ethics Committee of the Northern Rhine medical association (Ärztekammer Nordrhein) in Düsseldorf (application no. 2010-174). After providing a written informed consent, 28 healthy male subjects were included into the study and stratified according to their vertical jumping height into two matched groups with comparable neuromuscular fitness, using the maximum vertical jump height as an indicator[12]. A coin was then tossed to determine which group would perform either resistive vibration exercise (RVE) or resistive exercise (RE) only. The study was conducted in two campaigns due to feasibility reasons: the first campaign with 12 subjects took place between October 2010 and March 2011, the second campaign with 16 subjects took place between May and October 2011.

Participants and group design

Healthy, male subjects were targeted who were recreationally physically active (exercised 2-3 times per week). Any competitive sports, participation in strength training during the past six months, smoking, diabetes as well as any current medication were considered as exclusion criteria. Subject recruitment involved a telephone questionnaire checking for general suitability (224 applicants), a medical screening comprising a short medical history, blood analysis (involving a complete blood count and investigation of clinical parameters *-creatinin, urea, protein, albumin, SGOT, SGPT, γGT, Lipase, alk. phosphatase, electrolytes, glucose, C-reactive protein and haematological parameters: PTT, aPTT, Quick, INR*), as well as a urine test checking for glucose, protein and urobilinogen. Finally, a stress electrocardiogram on a cycling ergometer and a training familiarisation were performed. The medical screening involved 60 applicants out of which 28 were included in the study. The subject's anthropometric data at baseline are given in Table 1, and no statistically significant group difference was found ($P>0.08$).

	RE group (n = 13)	**RVE group (n = 13)**	***P*-value**
Age [yrs]	23.4 (± 1.4)	24.3 (±3.3)	0.52
Body mass [kg]	75.0 (± 4.7)	74.7 (±6.9)	0.08
Height [m]	1.79 (± 0.05)	1.79 (±0.05)	0.31
BMI	23.4 (± 1.4)	23.5 (±2.1)	0.11
CMJ height [cm]	42.2 (± 4.6)	41.7 (±2.2)	0.97
Maximal performance on cycle ergometer test [W/kg body weight]	3.3 (± 0.3)	3.3 (± 0.4)	1.00

Table 1. Anthropometric data of EVE subjects at baseline. BMI: Body Mass Index, CMJ: Counter movement jump. There was no difference between the two groups.

Training design

The present study was designed to compare acute and long-term effects of two training interventions: Resistive Exercise (RE) and Resistive Vibration Exercise (RVE). Subjects trained for six weeks, 2-3 times per week with additional weights. In order to align the squatting movement, the weights were put on a guided barbell (PTS Dual action Smith, Hoist, U.S.A). A vibration platform (Galileo® Fitness, Novotech, Germany) was placed underneath, as illustrated in Figure1A. The subjects in the RVE group performed the resistive exercise training protocol with simultaneous side-alternating whole-body vibrations, whereas subjects of the RE group trained with the same setting, without superimposed vibrations. We aimed to test physiological responses at 40 Hz side-alternating vibration, which has not been tested before. Preliminary testing yielded that this is challenging for people not acquainted with whole-body vibration. We therefore decided to initially set the vibration frequency to 20 Hz and to increase the vibration frequency throughout the study to eventually arrive at 40 Hz.

Estimation of training load

The individual training load was set at 80% of the subjects One-Repetition Maximum (1-RM), which was estimated in the familiarisation session four weeks prior to the first training, applying the method of Baechle and Earle[13] and performing squats in a non-vibrating condition.

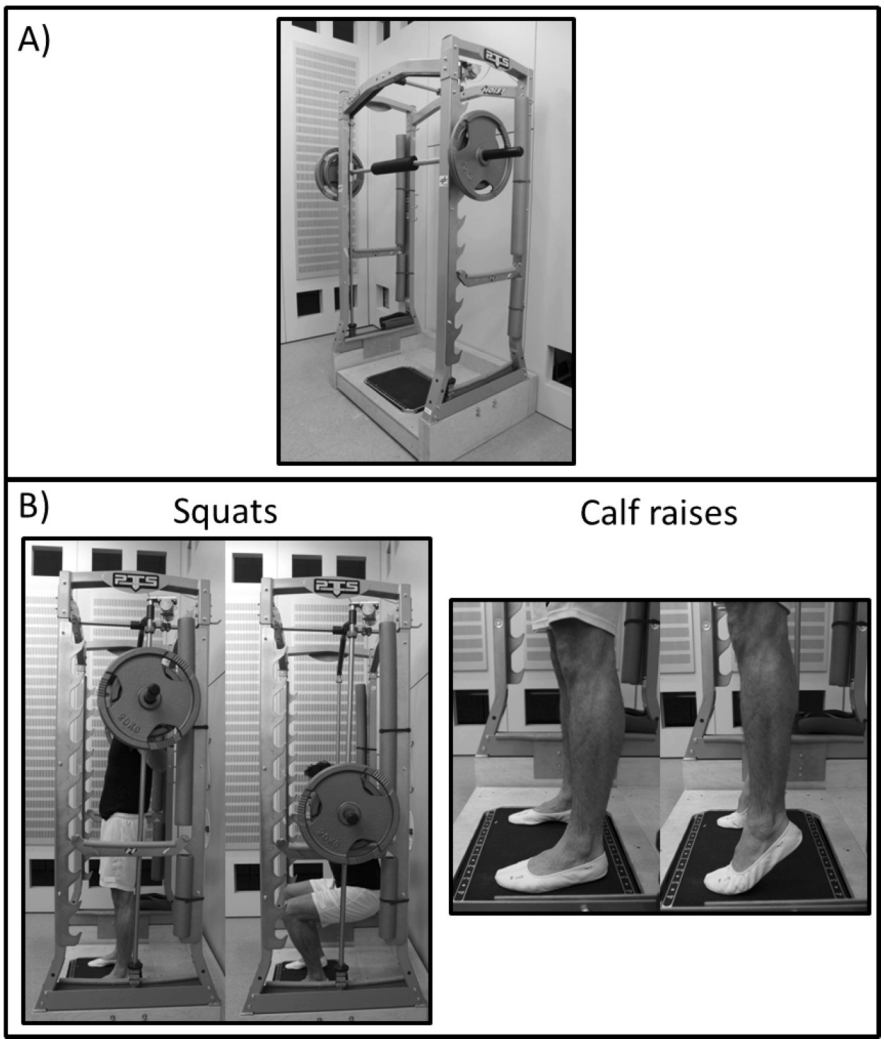

Figure 1. (**A**) Illustration of the training device. A guided barbell with a vibration plate placed underneath, embedded into a custom-built frame. (**B**) Illustration of the exercise movements. Squats (**left**) and calf raises (**right**).

% of the 1-RM	Repetitions
100	1
95	2
93	3
90	4
87	5
85	6
83	7
80	8
77	9
75	10

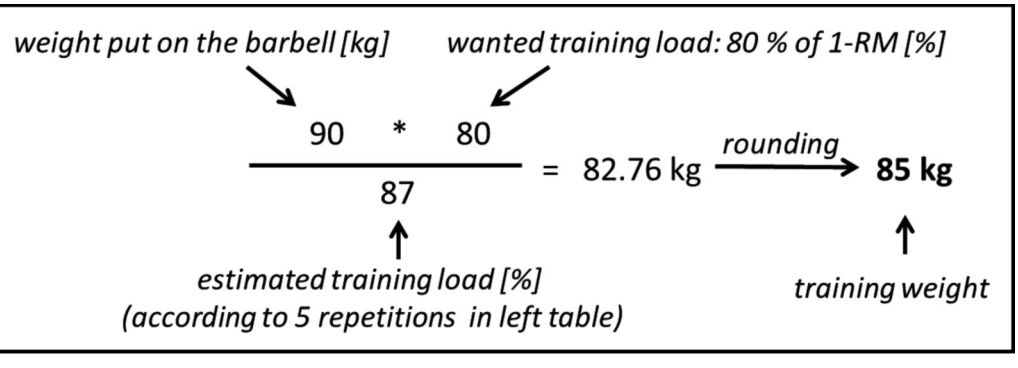

Figure 2. Determination of training load. **Left:** calculation of the performed % of the One-Repetition. Maximum (1-RM) according to the number of concluded repetitions (adapted from Baechle and Earle). **Right:** example for estimation of training load at 80 % of the 1-RM.

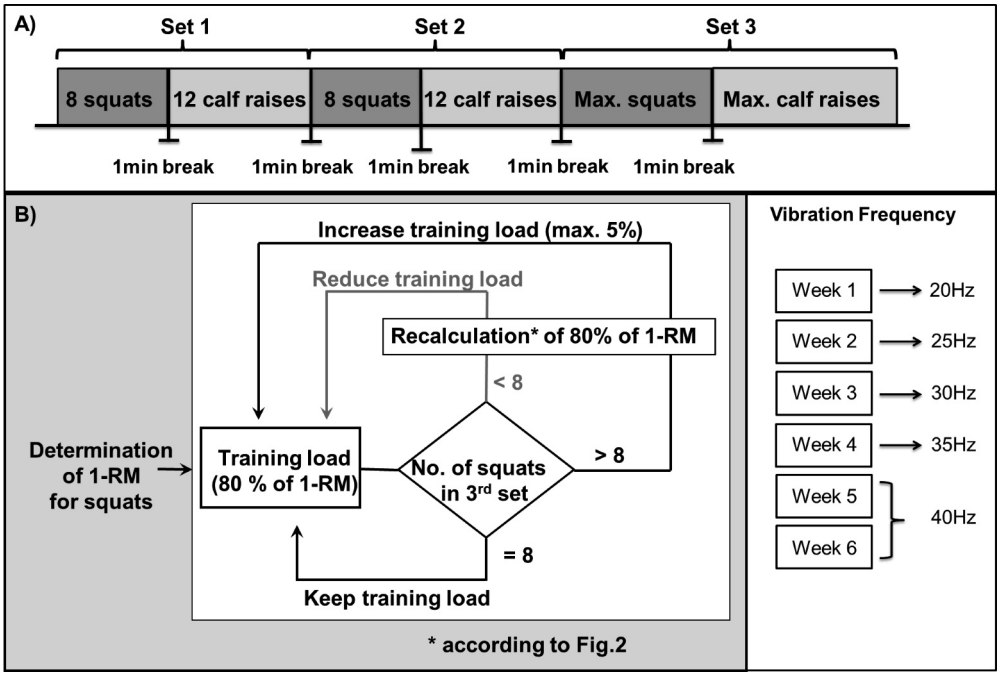

Figure 3. (**A**) Training design. After a warm-up, subjects performed three sets of squats and calf raises. The first two sets included 8 squats and 12 calf raises, in the third set, a maximum number of squats and calf raises was performed. (**B**) Increase of training intensity over the 6-week training intervention. **Left:** increase of training load for both intervention groups. **Right:** increase of vibration frequency in the resistive vibration exercise (RVE) group. 1-RM: One-Repetition Maximum.

Briefly, the guided barbell was initially loaded with weights corresponding to the subject's body weight plus 20 kg and subjects were asked to perform as many squats as possible. The corresponding % of the 1-RM was evaluated according to Baechle and Earle[13]. An example is illustrated in Figure 2: if the barbell was loaded with 90 kg and the subject's maximum number of repetitions was 5, which corresponds to 87% of the 1-RM, the training load was adjusted to 85 kg.

Training protocol

The training was supervised by a graduated exercise scientist throughout the study and two spotters were standing left and right of the guided barbell providing subject security. A metronome guided the training rhythm to provide standardisation of the movement. Squats were performed dynamically with 2 sec. eccentric and 2 sec. concentric phase; calf raises were performed with 1 sec. eccentric and 1 sec. concentric phase (Figure 1B). Each training session included a warm-up with the unloaded barbell (15 kg), which consisted of two sets; each set with 10 squats and 15 heel raises. The actual training was performed in three sets: the first two sets comprised 8 squats and 12 calf raises; in the third set, as many squats and calf raises as possible were performed (Figure 3A). Immediately after completion of the last set of squats, each subject's perceived exertion was rated via the Borg RPE Scale[14]. Altogether, the subjects concluded 16 training sessions in a period of 6 weeks (week 1-2: two sessions per week; week 3-6: three sessions per week). Both training regimens differed only in the vibration component.

Increase of training load and vibration frequency during the 6-week intervention

The number of squats in the third set was used to readjust the training weight to 80% of the 1-RM for the following training. When the number of squats in the third set was equal to 8, the training weight remained unchanged for the subsequent training. When the subjects performed more or less than 8 repetitions, the training weight was recalculated, i.e. increased or decreased for the next training. However, the top limit for weight increases was set at 10 kg in order to guarantee steady weight increments. The RVE group started the training with 20 Hz vibration with weekly increments by 5 Hz; during the last two weeks, vibration frequency was set at 40 Hz. A schematic overview of the incremental study design is displayed in Figure 3B.

Diet

During the initial and final training sessions, subjects ate a standardised breakfast two hours before training (two wheat bread rolls with butter and jam). During the long-term training intervention, subjects were asked to abstain from food two hours before every training session and to drink a protein energy drink (*Fresubin® protein energy drink,* Fresenius Kabi, Germany) one hour prior to training.

Study Week	-4	-3	-2	-1	1		2		3			4			5				6		+3d	+4d	+90d
Measurement / Training no.	BDC			Initial Training	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Final Training	Follow-up measurements		
Vibration frequency				20 Hz	20 Hz		25 Hz		30 Hz			35 Hz			40 Hz								
Diet (s=standardised, f=fastened)		f	f	s					f						f				f	s	f	f	
Protein Drink (1h pre training)					x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x			
Freiburg Questionnaire			x																		x		
Rating of Perceived Exertion				x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x			
Estimation of training load	x																						
Familiarisation Session	x																						
Body Weight Determination			x																		x		
Jump tests	x																				x		x
Doppler Ultrasound		x							x						x				x		x		
MRS		x																			x		x
MVC		x																			x		x
MRI			x																			x	x
Gait Analysis			x																x				
Blood Pressure, Heart Rate				x																x			
Lactate Test	x			x					x						x					x	x		x
Spirometry during Training				x																x			
EMG during Training				x																x			
NIRS during Training				x															x	x			
Serum Collection				x																x			
Muscle Biopsy			x	x																		x	

Figure 4. Overview of the EVE-Study design. BDC (Baseline Data Collection) was performed during 4 weeks prior to the initial training; follow-up measurements were performed 3, 4 and 90 days (d) after the final training. MRS: Magnetic Resonance Spectroscopy, MVC: Maximal Voluntary Contraction, MRI: Magnetic Resonance Imaging, EMG: Electromyography, NIRS: Near-Infrared Spectroscopy.

Measurements

The present study was designed to characterize the acute and long-term effects of resistive exercise and superimposed vibrations on both functional and molecular levels. An overview of the measurements with the corresponding time points is depicted in Figure 4.

Determination of daily physical activity

The Freiburg Questionnaire[15] was applied to assess the subject's daily physical activities. Subjects filled the questionnaire one week prior to and three days after the 6-week training intervention.

Blood pressure and heart rate at rest and during exercise

Resting heart rate and blood pressure were recorded after 20 minutes in horizontal position with an automated sphygmomanometer (Medicus pc, Boso, Germany). Exercise blood pressure was measured during each break between the sets and immediately after training termination by a medical doctor using a manual sphygmomanometer. Heart rate was measured manually by an exercise scientist.

Rating of perceived exertion (RPE)

The Borg RPE scale[14] was used for the assessment of the perceived exertion of the training. Within 20 sec after the last set of squats, subjects provided their individual RPE.

Statistical analyses

Statistical analyses were performed using STATISTICA 10 for Windows (Statsoft, Tulsa, Oklahoma, USA, 1984-2010). For estimation of differences in training load increments, rating of perceived exertion, blood pressure and heart rate, a repeated measures ANOVA was applied with time (initial vs. final) and intervention (resistive exercise vs. resistive vibration

Training week	Vibration Frequency	Back RVE	Pain RE	Headache RVE	Headache RE
1	20 Hz	-	-	-	-
2	25 Hz	-	-	-	-
3	30 Hz	3	1	1	-
4	35 Hz	1	1	2	-
5,6	40 Hz	4	-	-	-
	Sum	8**	2	3	0

Table 2. Important events during the study. Numbers of subjects are indicated perceiving headache or back pain in the respective training week. RE: resistive exercise group; RVE: resistive vibration exercise group. **Higher compared to RE group (chi-value<0.01).

exercise) as factors; Tukey's test was used for post-hoc testing. For estimation of daily physical activity (*Freiburg* Questionnaire), a paired, two-sided Student's t-test was performed to compare physical activity before and after of the 6-week training intervention; an unpaired, two-sided t-test was performed to test differences between the two intervention groups. For estimation of vibration-induced back pain, a chi-square analysis was performed. Values are given as means ± standard deviation, statistical significance was set at $P<0.05$.

Results

Freiburg Questionnaire of physical activity

Daily physical activities like walking, biking, stair climbing, activity at work, sleeping and weekly sportive physical activity did not differ before and after the 6-week training intervention (*P*-values between 0.12 and 0.96) and did not differ between the two intervention groups (*P*-values between 0.32 and 0.75).

Important events during the study

When training at frequencies above 30 Hz, eight of the RVE subjects complained about back pain. In one of the subjects, back pain was the cause for dropping out of the study. The sudden onset of back pain in the drop-out subject was caused by an incident during training. The impression of the personal trainer and his assistants present during that exercise session was that the incident resulted from training with poor body balance, which led to bending of the back. An independent orthopaedic surgeon diagnosed a facet joint syndrome L1-2, which did not implicate sensory or motor deficits. The back pain lasted for seven days after the incident and was ranked by the subject to an intensity of 8 using a scale ranging from 0 to 10, where 0 indicated "no pain" and 10 indicated "severe, unbearable pain". The subject had demonstrated questionable commitment before that event, which reinforced the decision was made to exclude him from the further participation.

Back pain reported by the other seven subjects that completed the study successfully was assessed via a questionnaire. All seven subjects reported low back pain without radiculopathy. One subject complained about pain during training, whereas the majority (6 out of 7 subjects) perceived back pain after training termination. The duration of the pain varied: two subjects reported acute pain until 1-2 hours after training, and four subjects reported pain until 2-3 days after training. The pain intensity estimated by the subjects ranged from 3 to 7 and was on average 4.4 (SD=1.4), using a 0-10 scale (as described above). None of the subjects had to take analgetics to relieve the pain. There were only two cases of back pain in the RE group: one subject complained about local neck pain at the site of weight application, the other subject complained about "light" muscle tenderness in the lumbar spine. Statistical analyses revealed that resistive vibration exercise at frequencies of 30 Hz and above caused back pain in a higher number of cases than resistive exercise alone (Chi-value<0.01); details are listed in Table 2.

Furthermore, four subjects in the RVE group complained about a training-induced headache with an onset after the second training set, out of which one subject dropped out after four weeks of training because of a headache that was reproducibly generated by the combination of vibration, application of the bar bell and calf raises. A post-hoc medical check revealed the absence of the physiological lordosis of the cervical spine as a likely explanation for this reaction.

Conduct of exercise: missed training sessions

In the RVE group, four subjects completed all 16 training sessions and nine subjects missed a single training session. In the RE group, ten subjects completed all 16 training sessions and three subjects missed a single training session.

Increase of training load

The training loads were comparable between the two groups at the initial training (RVE: 81.5±7.7 kg, RE: 75.2±6.5 kg; $P=1.0$) and increased over time in both groups ($P<0.001$). Compared to the initial training, the increase in training load over the six-week training intervention was significantly higher in the RE group and accounted for 59.8±17.3 %, compared to 46.9±19.0 % in the RVE group (time * intervention: $P<0.001$). As the weight increase was more pronounced in the RE group, post-hoc analyses reveal that RE subjects trained with significantly higher training loads compared to the RVE group in trainings 13 to 16 ($P<0.01$). During the final training, the RE group trained with 130.2±18.5 kg and the RVE group trained with 110.2±15.8 kg ($P=0.003$), see Figure 5.

Rating of perceived exertion (RPE)

The perceived exertion of the initial training was rated as "hard" according to the Borg RPE scale, and there was no difference between groups: 15.5±1.6 (RE) vs. 15.9±1.3 (RVE), $P=0.52$, see Figure 6. RPE data derived during the 6-week training reveal that superimposed vibrations did not alter RPE as there was no significant group effect ($P=0.73$). However, there was an overall increase in RPE over time ($P=0.048$). Post-hoc analyses showed that the RPE was higher during training 9-16 when compared to training 1-4 ($P<0.05$). During

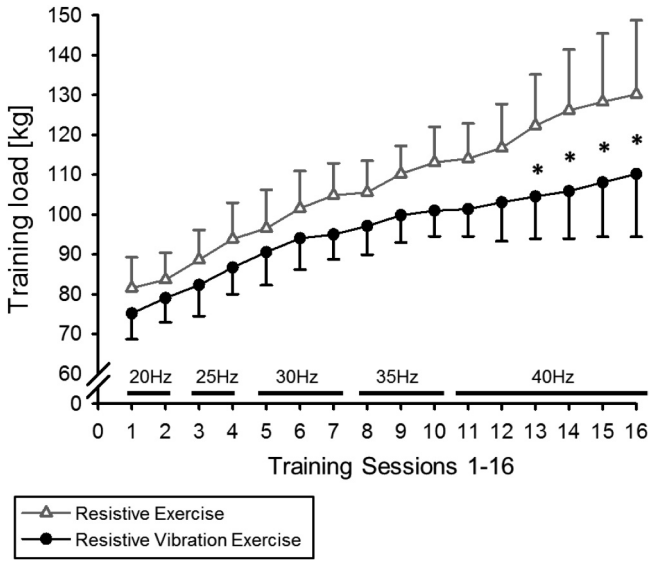

Figure 5. Training load increase during the 6-week training intervention. Bars indicating 20-40Hz refer to the applied vibration frequency in the RVE group. Training loads increased over time in both groups (time effect: $P<0.001$). The training load increase was more pronounced in the Resistive Exercise group and after the 13th training session, the RE group trained with significantly higher training loads (*$P<0.01$).

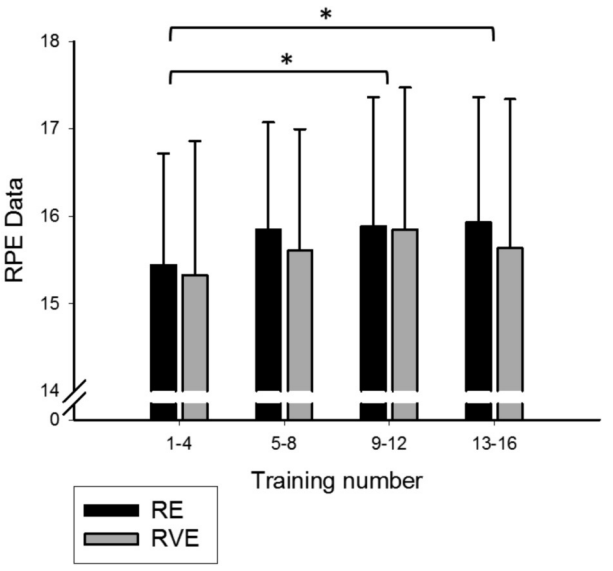

Figure 6. Rating of the training's perceived exertion. The subjects in both groups rated the perceivedexertion (RPE) of the training to "hard" and there was no difference between the Resistive Exercise (RE) and Resistive Vibration Exercise (RVE) groups. RPE was significantly higher in trainings 9-16 compared to trainings 1-4 (*$P<0.05$).

	Rest				During Training			
Group	**RE**		**RVE**		**RE**		**RVE**	
Variable	**Pre**	**Post**	**Pre**	**Post**	**Initial**	**Final**	**Initial**	**Final**
SBP [mmHg]	126 ± 8	118 ± 11**	122 ± 4	113 ± 9**	147 ± 18	143 ± 13	147 ± 13	142 ± 18
DBP [mmHg]	71 ± 9	65 ± 11	71 ± 6	62 ± 8**	81 ±8	72 ± 9 ***	82 ± 7	74 ± 10***
HR [bpm]	55 ± 9	52 ± 7	56 ± 8	54 ± 7	125± 17	127 ± 15	126 ± 21	131 ± 23

Table 3. Cardiovascular parameters at rest (left) and during exercise (right). Stars indicate significant difference (time effect) within the same group: *$P<0.05$; **$P<0.01$; ***$P<0.001$. *Pre* and *Post* refer to resting values before and after 6 weeks of training; *Initial* and *Final* Training refer to the first and last exercise session of the 6-week training intervention. SBP: systolic blood pressure; DBP: diastolic blood pressure; HR: heart rate.

the last training, RPE accounted for 15.9 in the RE group and 16.38 in the RVE group. Of note, RPE during the last training was comparable between groups ($P=0.15$), although the RE group trained with significantly higher training loads ($P=0.003$). Furthermore, there was no correlation between RPE and heart rate (R=-0.13; $R^2=0.017$; $P=0.42$) as previously described for endurance exercise[16].

Cardiovascular parameters at rest

Resting Systolic Blood Pressure (SBP) and Diastolic Blood (DBP) pressure were both decreased from pre levels during the follow-up measurement after 6 weeks of training (SBP: $P=0.003$; DBP: $P=0.001$) with no significant differences between the two groups (SBP: $P=0.06$; DBP: $P=0.5$) as depicted in Table 3. Post-hoc analyses revealed that the decrease of DBP was more pronounced in the RVE group as this group depicted significant decreases ($P=0.01$), whereas the decrease of DBP did not reach significance in the RE group ($P=0.055$). Resting heart rate (HR) remained unaffected by the training intervention in both groups ($P=0.14$), see Table 3.

Cardiovascular parameters during exercise

Blood pressure and heart rate measured within single training sessions were comparable between sets ($P>0.28$) and therefore, data of the three sets were pooled for further analysis. There was a trend of decreased systolic blood pressure during exercise after 6 weeks of training in both groups, which however failed to reach the level of significance ($P=0.052$). Diastolic blood pressure during exercise was significantly decreased in both groups ($P<0.001$). As a result of the decreased

DBP with unaltered SBP, exercise pulse pressure (=SBP-DBP) was significantly increased in both groups after 6 weeks of training (P=0.04). Six weeks of training did not alter exercise heart rate in neither of the groups (P=0.39), see Table 3. Exercise blood pressure and exercise heart rate did not differ when comparing RE to RVE (SBP: P=0.9; DBP: P=0.6; HR: P=0.5).

Discussion

Feasibility

The incremental design of the training was reflected by an increase in Borg RPE over time (Figure 6), as the training was perceived as significantly "harder" in training sessions 9-16 compared to training sessions 1-4. The subject's daily physical activities were comparable between the two groups and did not change over the duration of the study (*Freiburg* Questionnaire). These data indicate that the obtained results from the EVE study actually derive from the training intervention itself and were not induced by external factors.

While vibration frequency was increased on a weekly basis, the RVE group trained at equal or higher training loads compared to the previous week. Only in four cases out of 52 individual increases in vibration frequency (=4 frequency increases * 13 subjects), training loads had to be decreased due to an increase in vibration frequency when training with frequencies above 35 Hz. When training with frequencies between 20 and 30 Hz, superimposed vibrations were well tolerated. However, data from the present study suggest that the risk of low back pain is substantially increased when performing resistance exercise with superimposed vibrations and frequencies above 30 Hz (see Table 2). Seven out of thirteen subjects that concluded the study successfully complained about low back pain, which would probably be classed as uncomfortable, but not severe. The back pain might either derive from the vibration itself, or from the way that the guided barbell was employed, which was always with a certain reclination toward the back. This could have increased the amount of instability in the movement when training with high vibration stimulation. This lack of stability might have caused the training incident that led to the drop-out of one subject in the RVE group. However, it remains unknown whether the vibration component was actually the cause for the training incident.

Demands

Increase of training load with and without superimposed vibrations

There was no difference between the two groups concerning One-Repetition Maximum or jump height at the beginning of the study, indicating two groups with comparable muscular performances. As expected, training loads were increased over time. However, after the 13th training session, when RVE subjects trained with 40 Hz simultaneous vibrations, the increase of training weight was hampered (Figure 5) compared to the group training without vibrations. In the end of the study, the RE group trained at 18% higher training loads compared to the RVE group. It is known that sinusoidal vibrations engender increases in peak foot acceleration to the power of two[10], and thus, increases in vibration frequency lead to pronounced elevations of musculoskeletal forces. We conclude from our data that the increase of training weight (*external* training load) might be hampered by vibration-induced elevation of musculoskeletal forces (*internal* training load) and the combination of the two add up to the total muscle loading during RVE. This idea is supported by the Rating of Perceived Exertion data, which indicate that training at lower weights with 40 Hz WBV was perceived equally demanding as training without vibrations and higher weights.

Chronic cardiovascular adaptations at rest

There is strong evidence supporting beneficial effects of endurance exercise upon cardiovascular health such as decreases in blood pressure and heart rate[1,2]. However, limited data are available on the effect of long-term resistance exercise training in healthy, recreationally active people. Resistance exercise has been reported to have beneficial effects in obese subjects as well as in people with metabolic syndrome or hypertension[17-19]. Previous studies involving healthy young males show that resting systolic and diastolic blood pressures were decreased by a resistance training intervention[8,20]. Another study shows a 4% decrease in resting systolic with no change in diastolic blood pressure[5]. Results from the present study show that resting systolic and diastolic blood pressures were both decreased by 7 to 12 % after only six weeks of training and there were no alterations in resting heart rate. Our data support the view that high-resistance exercise is beneficial for cardiovascular health. Further, our data suggest that superimposed vibrations might be additionally beneficial as diastolic blood pressure was significantly decreased only in the RVE group.

Chronic adaptations of the acute cardiovascular responses to resistance exercise

It has been shown that body builders have lower systolic and diastolic blood pressures and heart rates during resistance exercise compared to recreationally active people[21]. Previous studies have reported that resistance training results in adaptations that hamper the acute training-induced increases in heart rate and blood pressure[22,23]. In the current study, we found that 6 weeks of resistive exercise decreased diastolic blood pressure during exercise whereas systolic blood pressure and heart rate were unaltered compared to the initial training. This decrease in diastolic blood pressure might derive from increased vasodilation during exercise and thus, the applied training interventions in the current study seem to have improved vascular responsiveness. This idea is supported by previous studies showing that WBV increases blood flow velocity after vibration termination[24,25], indicating vibration-induced dilation of feeding arteries. Our data reveal that only exercising diastolic blood pressure was decreased after 6 weeks of training, whereas systolic blood pressure remained unaltered, yielding increases in pulse pressure (=SBP-DBP). As pulse pressure is known to be proportional to stroke volume[26], there is evidence that the resistive exercise intervention conducted in this

study increased cardiac stroke volume and maybe cardiac output. There was, however, no additional effect of superimposed vibrations, neither during the first training nor after 6 weeks of training.

Summary and Conclusions

In summary, both training interventions were feasible and the incremental training design was reflected by an increase in RPE. Superposition of vibrations to resistive exercise for some reasons hampered the increase of training load when training at frequencies above 35 Hz. Furthermore, our data show that 6 weeks of resistance exercise decreased resting blood pressure (systolic and diastolic) as well as exercising diastolic blood pressure. We conclude that WBV in combination with high-resistance exercise is well tolerated when training with frequencies below 35 Hz. However, when training with 35 Hz and above, this exercise type seems to foster back pain and to reduce training performance. It is possible that training with side-alternating vibration above 30 or 35 Hz may elicit sub-optimal results. Thus, it might not be recommendable to use these high frequencies combined with resistance exercise, at least not for non-athletes. Finally, our data also demonstrate a beneficial effect upon arterial blood pressure.

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6.3 Curriculum Vitae

Åsa Beijer was born in Heidelberg, Germany, in 1982. In 2001 she finished high school with the degree 'Abitur' and studied economics and romanic languages at the University of Heidelberg until February 2002. From March until August 2002, she studied Media and Communication at the University of Uppsala, Sweden. In October 2002, she started her studies in Biology at the University of Cologne, which she completed in April 2008. Her diploma thesis entitled 'Sun-1 tethers the novel KASH domain protein Emerin at the nuclear envelope' was conducted in the Institute for Medical Biochemistry at the University of Cologne. From June until September 2008, Åsa underwent a further training in clinical research at the 'Mibeg-Institute for Medicine' in Cologne and from October 2008 until April 2009 she did an internship at the contract research organization 'Keypoint' in Lisbon, Portugal. From May until July 2009, she became a graduate assistant in the Biomedical Support Center of the German Aerospace Center (DLR), Cologne. In September 2009, she received the PhD fellowship 'SpaceLife' which is funded in equal parts by the Helmholtz Association and the DLR. She did her PhD in the Space Physiology Department of the DLR Institute of Aerospace Medicine, led by Prof. Jörn Rittweger and in the Institute of Cardiovascular Research and Sport Medicine, Department of Molecular and Cellular Sport Medicine at the German Sport University Cologne, led by Prof. Dr. Wilhelm Bloch.