Exercise training in patients with advanced chronic heart failure (NYHA IIIb) promotes restoration of peripheral vasomotor function, induction of endogenous regeneration, and improvement of left-ventricular function

Running Title: Exercise training and regenerative capacity in CHF

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Journal Subject Codes: 110; 26; 129; 95
ABSTRACT

Background: Attenuated peripheral perfusion in patients with advanced chronic heart failure (CHF) is partially the result of endothelial dysfunction. This has been causally linked to an impaired endogenous regenerative capacity of circulating progenitor cells (CPC). Aim of this study was to elucidate, whether exercise training (ET) affects exercise intolerance and left-ventricular (LV) performance in patients with advanced CHF (NYHA IIIb) and whether this is associated with correction of peripheral vasomotion and induction of endogenous regeneration.

Methods and Results: Thirty-seven patients with CHF (LVEF 24±2%) were randomized to 12 weeks of ET or sedentary lifestyle (control, C). At beginning and after 12 weeks, maximal oxygen consumption (VO2max) and LVEF were determined; number of CD34+/KDR+ CPCs was quantified by flow cytometry and CPC functional capacity was determined by migration assay. Flow-mediated dilatation (FMD) was assessed by ultrasound. Capillary density was measured in skeletal muscle (SM) tissue samples. In advanced CHF, ET improved VO2max by +2.7±2.2 vs. -0.8±3.1 mL/min/kg in C (p=0.009) and LVEF by +9.4±6.1 vs. -0.8±5.2 % in C (p<0.001). FMD improved by +7.43±2.28 vs. +0.09±2.18% in C (p<0.001). ET increased the number of CPC by +83±60 vs. -6±109 cells/mL in C (p=0.014) and their migratory capacity by +224±263 vs. -12±159 CPC/1000 plated CPC in C (p=0.03). SM capillary density increased by +0.22±0.10 vs. -0.02±0.16 capillaries/fiber in C (p<0.001).

Conclusions: Twelve weeks of ET in patients with advanced CHF is associated with augmented regenerative capacity of CPCs, enhanced FMD suggestive of improvement in endothelial function, SM neovascularization, and improved LV function.

Clinical Trial Registration: http://www.clinicaltrials.gov. Unique Identifier: NCT00176384.

Keywords

heart failure; exercise; hemodynamics; endothelium; progenitor cells
INTRODUCTION

Limitation of exercise capacity in patients with advanced chronic heart failure (CHF) is not only due to impairment of left ventricular (LV) function but also a result of peripheral maladaptations involving an impaired peripheral perfusion secondary to endothelial dysfunction and intrinsic alterations of skeletal muscle, e.g. a reduction in capillary density. In the past, endothelial dysfunction in CHF was primarily attributed to the imbalance between the production of nitric oxide (NO) by the endothelial isoform of the nitric oxide synthase (eNOS) and the rapid inactivation of NO by reactive oxygen species that are generated by a wide variety of enzymes in excessive amounts. However, excessive generation of reactive oxygen species does not only lead to a rapid NO inactivation, but also promotes endothelial cell apoptosis, thereby obliterating the integrity of the endothelium and attenuating its function. In the past, mature endothelial cells bordering the area of damage were thought to be responsible for vascular repair. Nevertheless, recent data suggest that bone marrow-derived progenitor cells (CPCs) restore diseased endothelium, but their functional capacity appears to be impaired in advanced stages of CHF due to a general inflammatory activation.

In patients with stable, moderate CHF (NYHA II), exercise training has been shown to enhance exercise capacity and to partially reverse intrinsic alterations of the skeletal muscle in the absence of harmful side effects on central hemodynamics.

In contrast, the therapeutic benefits of regular exercise training in patients with advanced CHF - that fulfill the inclusion criteria of the COPERNICUS trial - are not established yet. Therefore, it was the aim of the present trial to elucidate whether regular physical exercise training improves exercise capacity in patients with advanced CHF, and whether this is the result of 1) enhanced endogenous regenerative capacity, 2) a restoration of peripheral vasomotor function, and 3) an improvement in LV performance.
METHODS

This trial is registered at http://www.clinicaltrials.gov with the following number: NCT00176384.

Patient selection

This prospective, randomized controlled study was conducted in accordance with the principles of good clinical practice and the Declaration of Helsinki. The protocol was approved by the Ethics Committee of the University of Leipzig (votum number 157/2002), and written informed consent was obtained from all patients.

The study consisted of 37 male patients ≤ 70 years with CHF as a result of ischemic heart disease or dilative cardiomyopathy as assessed by cardiac catheterization. All patients had clinical signs of CHF according to NYHA functional class IIIb, a LV ejection fraction ≤ 30 % and a LV end-diastolic diameter ≥ 60 mm as determined by echocardiography. They were required to be clinically stable for at least 2 months before enrollment into the study and had to have a peak oxygen uptake ≤ 20 mL/min/kg body weight. Patients with CHF received their individually tailored medication consisting of angiotensin-converting enzyme inhibitors or AT1 blockers, beta-blockers, aldosterone antagonists, and other diuretics. Exclusion criteria were insulin-dependent diabetes mellitus, untreated arterial hypertension, untreated hyperlipidemia, active smoking, cardiac decompensation within the last 4 weeks, significant valvular heart disease, and unprotected ventricular arrhythmias, respectively.
Eligible patients were randomly assigned in a 1:1 ratio to undergo either 12 weeks of exercise training (training group) or sedentary lifestyle (control group) using computer-generated random numbers.

Training protocol

The initial phase of the exercise program was supervised and performed in-hospital. During the first 3 weeks, patients exercised 3 to 6 times daily for 5 to 20 minutes on a bicycle ergometer adjusted to the work load at which 50% of maximum oxygen uptake (VO₂max) was reached. Before discharge from hospital, symptom-limited spiroergometry was performed again to determine the training target heart rate for home training (defined as the heart rate reached at 60% of VO₂max). Upon discharge, patients were provided with bicycle ergometers for home exercise training. They were encouraged to exercise close to their target heart rate daily for 20-30 minutes for a period of 12 weeks and were expected to participate in one supervised group training session for 60 minutes each week consisting of walking, calisthenics and non-competitive ball games. Patients assigned to the control group continued their sedentary lifestyle.

Exercise testing and respiratory variables, measurement of peripheral endothelial function and echocardiography

Vasoactive medications were discontinued 24 hours prior to the measurements at begin and after 12 weeks in all patients of both groups. Exercise testing was performed on a calibrated, electronically braked bicycle in an upright position with work load increasing progressively every 3 min in steps of 25 W beginning at 25 W. Respiratory gas exchange data were determined continuously throughout the exercise test 11.
Flow-mediated dilatation (FMD) of the radial artery was measured using a high-resolution ultrasound scanning echo-tracking angiometer (NIUS 02, Asulab Research Laboratory, Neuchâtel, Switzerland) as described previously\(^3,12\). Quantification of radial artery internal diameter is well established and validated in our laboratory\(^3,12\). One experienced investigator blinded to patient identity, group assignment, and intervention status analysed FMD recordings after completion of the study.

All patients underwent a complete echocardiography at study begin and at 12 weeks according to guidelines. LV diameters, volumes, and ejection fraction were acquired as an average from 5 consecutive beats according to guidelines for two-dimensional echocardiography\(^13\). Only one experienced cardiologist, who was blinded to patient identity, group assignment, and intervention status performed all echocardiographic analyses.

**Skeletal muscle biopsies**

Two days before maximal spiroergometry at baseline and after 12 weeks percutaneous needle biopsies (approximately 50 mg/ biopsy) were obtained from the middle part of the *vastus lateralis* muscle under local anesthesia\(^14\). The biopsies were either snap frozen in liquid nitrogen and stored at –80°C or fixed with 4% buffered formaldehyde before paraffin embedding.

**Measurement of number and function of circulating progenitor cells**

At beginning and at 12 weeks, the number of CPCs was analyzed in venous blood by flow cytometry using the following antibodies: anti-human KDR (R&D Systems, Wiesbaden, Germany) and anti-human CD34 (Miltenyi, Bergisch Gladbach, Germany). Functional capacity of CPCs was determined by migration assay as described previously\(^15\).
Measurement of circulating growth/homing factors, systemic markers of inflammation and oxidative stress

Plasma levels of vascular endothelial growth factor (VEGF), stromal-derived factor 1 (SDF-1), tumor necrosis factor alpha (TNFalpha), and lipid peroxides (LPO) were measured by highly sensitive ELISA (VEGF, SDF-1, TNFalpha: R&D-Systems, Wiesbaden, Germany; LPO: Immundiagnostik, Bensheim, Germany).

Measurement of neovascularization of skeletal muscle

*Identification of CD34*⁺* cells in the skeletal muscle:* CD34⁺ cells were identified in paraffin sections of skeletal muscle by immunohistochemistry using as specific antibody (Dako, Hamburg, Germany). The density of premature capillaries containing CD34⁺ cells is expressed as number of CD34⁺/high power field (hpf).

*Identification of von Willebrand factor (vWF⁺) capillaries:* Mature capillaries were identified in paraffin sections of skeletal muscle by an anti-human vWF antibody (Dako, Hamburg, Germany). The density of mature capillaries was calculated as the number of vWF⁺ vascular structures/muscle fiber¹.

Statistical analysis

Primary endpoint of the overall study was the change in VO₂max. Since a NYHA III population has never been investigated so far, we estimated a 20% less treatment effect with regard to VO₂max compared to one of our previously published training trials with pre-dominantly NYHA II patients². Therefore, we assumed a treatment effect of exercise on VO₂max of + 3.8 mL/min/kg with a SD of 3.8. With an error of 0.05 and a error of 0.20 (power 80%), minimal group size was estimated to be 17 patients in each group.
Secondary endpoints of the study are training-induced changes in LV function and size, FMD, regenerative capacity of CPCs, NYHA class functional status, and skeletal muscle as well as serum markers.

Mean value ± standard deviation (SD) was calculated for all variables. Analysis of variance testing was used to compare the incidence of the primary end-point – the change in FMD – between the groups. Comparisons from baseline characteristics were performed using an analysis of covariance (ANCOVA) model. All other secondary analyses were performed using a Mann Whitney U test or t-test, where appropriate. Categorical variables were tested applying the Chi-Square or Fishers exact test. A p-value of less than 0.05 was considered statistically significant.

All investigators and lab staff involved in the data analysis were blinded to patient identity, group assignment, and intervention status.

The authors had full access to the data and take responsibility for its integrity. All authors have read and agreed to the manuscript as written.

For the readers convenience, a detailed description of the methodology is provided in the online Data Supplement.
RESULTS

Baseline characteristics and clinical follow-up

From March 2003 to December 2006 a total of 37 patients were enrolled. Eighteen patients were randomized to the training group, whereas 19 patients remained sedentary as a control group (Figure 1). At baseline, patients in both groups did not differ with respect to age, etiology and duration of CHF, ejection fraction, VO$_2$max, and the number of implanted cardioverters/defibrillators. History of atrial fibrillation was reported in 56% of patients in the training group and 47% in the control group, respectively. However, only 17% (training) and 5% (control) had atrial fibrillation during the study course. Six patients in each group had cardiac cachexia. Medical therapy was similar in both groups and did not change during the study period (Table 1). One patient in each group withdrew his consent for study participation within the first weeks after enrollment. The medication and baseline characteristics of those patients did not differ from those who successfully participated in the entire study. One patient in the control group died from sudden cardiac death. Within the 12 weeks of the study period, there were no differences between the training and control groups with regard to serious adverse events, including cardiac decompensations, hospitalizations due to worsening of heart failure, revascularization procedures, acute myocardial infarction or life-threatening ventricular arrhythmias.

Exercise capacity and clinical symptoms

At baseline, VO$_2$max as a measure of exercise capacity was severely reduced in both groups. Following patients self reports and training logs as well as the analysis of the memory chips from the bicycle ergometers, the training compliance was estimated to be appr. 90%. The training program led to an increase in VO$_2$max from 15.3±3.3 at begin to 17.8±3.2 mL/min/kg at 12 weeks (p=0.001 versus control) in the training group. Oxygen uptake at the ventilatory threshold...
improved after training from 11.9±2.6 to 14.2±2.7 mL/min/kg (p=0.009 versus control). This was associated with a decline in NYHA functional class from 3.0±0.0 before to 1.8±0.4 at 12 weeks in the training group (p<0.001 versus control). All of the above-mentioned parameters remained stable in the control group (Table 2). Also NYHA functional class remained nearly unchanged in the control group (3.0±0.0 before to 2.8±0.4 at 12 weeks). There were no changes in heart rate, diastolic or systolic blood pressure at rest during the study period, neither in the control nor in the training group (Table 2).

Left ventricular performance

The exercise training program resulted in a decrease in LV end-systolic diameter from 60.0±7.3 at begin to 51.3±7.8 mm at 12 weeks (p<0.001 versus control), whereas LV end-diastolic diameter declined from 69.5±7.5 before to 62.6±6.8 mm at 12 weeks (p<0.001 versus control). This was associated with a training-induced reduction of LV end-systolic and end-diastolic volume, respectively (Table 3). Resting LV ejection fraction increased from 24.1±5.1 before to 33.5±5.7 % at 12 weeks in the training group (p<0.001 versus control) and resting fractional shortening was found to be augmented, too (p=0.003 versus control). LV end-diastolic diameter and volume, resting LVEF and fractional shortening did not change significantly during the study period in the control group (Table 3).

Peripheral endothelial function

At baseline, radial artery FMD was significantly blunted in both groups. At begin, internal diameter increased during reactive hyperemia by 186±69 mm and by 181±82 µm in the training and control group. Exercise training for a period of 12 weeks resulted in a significant
improvement in FMD from $6.1\pm2.5\%$ to $13.6\pm2.2\%$ (absolute increase in internal diameter of $415\pm86\ \mu m$) after 12 weeks of exercise training ($p<0.001$ vs. control), and hence a complete normalization of peripheral vasomotion in the training group. In contrast, FMD did not change in the control group (absolute change in internal diameter $+185\pm71\ \mu m$ after 12 weeks; Figure 2).

**Number and functional properties of CPCs**

Exercise training over 12 weeks increased the number of CD34$^+$ progenitor cells from $1094\pm677$ to $1450\pm798$ cells/mL blood in the training group ($p=0.032$ versus control). The number of CD34$^+$/KDR$^+$ CPCs was found to be augmented from $100\pm127$ to $183\pm156$ cells ($p=0.014$ versus control) as a result of the exercise training intervention. CPC migratory capacity is paramount to their ability to home to ischemic tissues. Exercise training for a period of 12 weeks improved the ability of CPCs to follow a SDF1 gradient through a semi-permeable membrane from $211\pm102$ to $437\pm267$ CPCs/1000 plated CPCs ($p<0.001$ versus control). All of these parameters remained virtually unchanged in the inactive control group (Figure 3b and c).

**Capillary density in the skeletal muscle**

Physical exercise training for a period of 12 weeks increased the number of vWF-expressing mature capillaries from $1.49\pm0.11$ to $1.71\pm0.11$ capillaries/fiber ($p<0.001$ versus control), whereas no change was observed in the control group ($1.50\pm0.13$ capillaries/fiber at begin, $1.48\pm0.11$ capillaries/fiber at 12 weeks), (Figure 4a).

Additionally, density of CD34$^+$ stem cells that might contribute to neovascularization increased from $5.81\pm1.44$ to $7.26\pm1.36$ cells/hpf ($p<0.01$ versus control) in patients of the training group. No significant change in the density of CD34$^+$ cells was observed in the control group group ($6.63\pm2.52$ cells/hpf at begin, $6.38\pm2.91$ cells/hpf at 12 weeks), (Figure 4b).
Circulating growth and homing factors

Since training has been suggested to promote neovascularization in part through activation of growth factors, circulating VEGF levels were quantified. Twelve weeks of exercise training significantly increased plasma VEGF from 286±213 to 364±258 pg/mL (p=0.02 versus control, Figure 3a). Additionally, serum SDF-1 concentration increased in the training group from 2247±341 to 2881±335 pg/mL (p<0.001 versus control). In the inactive control group, circulating VEGF level (Figure 3a) and SDF-1 concentration (2381±390 pg/mL at begin, 2324±279 pg/mL at 12 weeks) remained nearly unchanged.

Systemic oxidative stress and inflammation

The 12 week exercise training program exerted significant antioxidative and anti-inflammatory effects, since it reduced LPO levels from 271±130 to 193±107 pg/mL and decreased TNFalpha concentration from 9.8±6.3 to 7.3±4.6 pg/mL (p=0.011 versus control). Both parameters remained stable in the control group (LPO: 272±180 pg/mL at begin vs. 227±124 pg/mL at 12 weeks; TNFalpha: 9.1±5.5 at begin vs. 10.4±6.2 pg/mL at 12 weeks).
DISCUSSION

Impact of exercise training on exercise capacity

Despite of the compelling evidence that exercise training is beneficial in moderate CHF, only few data are available about the impact of exercise training on patients with advanced CHF. The lack of information is basically related to the fact that patients with advanced CHF were mostly excluded from study participation in previous trials. The reluctance of physicians to propose regular exercise training to patients with severe CHF might have its origin in theoretical considerations that acute bouts of exercise can cause cardiac decompensations or life-threatening arrhythmias. However, a recently published retrospective analysis suggests that also patients in advanced stages of CHF did benefit from exercise training with regard to hemodynamics and exercise capacity\textsuperscript{16}.

To our knowledge, this is the first, randomised trial that determined the impact of regular, aerobic exercise training on exercise capacity and LV performance in patients with advanced CHF that would fulfill the inclusion criteria of the COPERNICUS trial\textsuperscript{10}, since in all previously published studies NYHA III patients were only small subgroups and the respective treatment effects underpowered. Although in the HF-action trial the total number of NYHA III patients was 800 (~one third of patients), it is difficult to conclude a comparability between this and our study, because the training intensity was completely different (~200-250 kcal consumption/ week compared to ~650 kcal/ week in our trial). This major difference may also explain at least partially, why HF-action failed to demonstrate beneficial effects with regard to outcome\textsuperscript{17}.

This study shows for the first time, that patients with advanced CHF still benefit from a regular, aerobic exercise training program: the severity of dyspnoea improved by at least one NYHA class in all patients after exercise training, and VO$_2$max increased by 16% after only 12 weeks of
regular training. These beneficial effects occurred in the absence of life-threatening arrhythmias or an increased number of cardiac decompensations.

Impact of exercise training on left ventricular performance

In the past, it has been shown that regular physical exercise training leads to a modest but significant decline in cardiac size and an improvement in LV function in patients with moderate CHF\(^2\). Nevertheless, in the present trial it was intriguing to see that exercise training might be even more effective in patients with advanced CHF: patients in the training group gained an absolute value of about 10 % in ejection fraction after 12 weeks of exercise training, which is about twice as much seen after 6 months of cardiac resynchronisation therapy\(^18\). The positive effects of exercise training on LV performance were not associated with a further LV dilatation, the opposite was the case: LV end-diastolic and end-systolic diameter and volume declined significantly. These data clearly suggest that aerobic exercise training might have the potential to partially reverse adverse LV remodelling not only in patients with moderate CHF\(^19\).

However, the question raises which mechanisms are responsible for the exercise training-mediated decline in cardiac size and increase in ejection fraction. We and others have previously shown that exercise training leads to a partial correction of peripheral endothelial dysfunction in patients with moderate CHF\(^3\)\(^,\)\(^20\). Given that vascular tone of peripheral arteries is one component of afterload, it is obvious that the improvement in endothelial function observed after aerobic endurance exercise training was inversely correlated to the decline in systemic vascular resistance in a previous study\(^2\). A training-induced improvement of endothelial function was also detectable in the present trial. Therefore, it is plausible to assume that the reduction in cardiac size and improvement in LV performance is at least partially the result of an exercise training-induced decline in afterload.
However, it is alternatively conceivable that exercise training exerts beneficial effects on cardiomyocytes itself. Recent experimental studies suggest that exercise training might have the potential to increase cardiac contractility, enhance calcium sensitivity and calcium cycling by phosphorylation of Ca\(^{2+}\)/calmodulin-dependent protein kinase II and Threonine-17 of phospholamban\(^{21}\). Moreover, exercise training is known to enhance phosphorylation of the survival kinase AKT at serine 477, which is associated with an augmentation in cardiac contractility\(^ {22,23}\). In addition, exercise training might re-adjust the balance between anabolic and catabolic processes in the heart: the TNFalpha, that is overexpressed at the myocardial level in CHF, activates the expression of the E3-ligase MuRF1 – a key regulator in the ubiquitin-proteasom-pathway – and in turn induces troponin I ubiquitinylation, and subsequent degradation, resulting in a decline in contractility in experimental CHF. The activation of this deleterious cascade was recently shown to be partially prevented by exercise training\(^ {24}\).

**Modulation of peripheral vasomotion by exercise training: importance of endogenous progenitor cells**

Endothelial dysfunction is a common feature in patients with CHF and a predictor of future cardiovascular events\(^ {25}\). Given the prognostic value of endothelial dysfunction, it became a target of therapeutic interventions. In this regard, exercise training appears to be the most powerful and promising therapy since it completely normalized FMD in the present population of patients with advanced CHF. The molecular alterations leading to endothelial dysfunction are multifarious and include an increase in oxidative stress due to an upregulation of radical producing enzymes, e.g. NAD(P)H oxidase, xanthine oxidase or an uncoupled NOS, a decline in the activity of radical scavenger enzymes, a reduction in eNOS expression and phosphorylation which in turn results in a decline in NO bioavailability\(^ {4,6}\). However, exercise training has the potential to reverse a part of
these alterations in patients with cardiovascular disease since it reduces the expression of the NAD(P)H oxidase and enhances eNOS expression as well as phosphorylation\textsuperscript{23,26}. This leads to a reestablishment of the balance between the NO production and inactivation by reactive oxygen species and hence, an improvement of vasomotion.

The increase in oxidative stress does not only lead to the premature breakdown of NO, it also induces the death of endothelial cells by apoptosis in CHF. The disruption of the integrity of the endothelial layer is associated with a further aggravation of vascular dysfunction and might finally lead to the development of atherosclerotic plaques. Recent studies suggest that circulating bone marrow-derived progenitor cells have the capability to rejuvenate diseased endothelium\textsuperscript{8} but their number was found to be impaired in advanced CHF, most likely due to the myelosuppressive effects of TNFalpha\textsuperscript{6}. In the present study, we were able to show that exercise training exerts anti-inflammatory effects such as the reduction of systemic TNFalpha levels. Besides lowering the pro-apoptotic impact on resident endothelial cells, reduced concentrations of TNFalpha might also explain the increase in CPC number and function we found in the exercise training group. Moreover, it is conceivable that the exercise training mediated augmentation in vascular NO production contributes to an activation of matrix metalloproteases, which are known to mediate the transition of progenitor cells from the silent to the proliferative niche\textsuperscript{27}. Increased plasma levels of SDF-1 and VEGF, as seen in the training group, might furthermore facilitate CPC liberation from the bone marrow.

**Impact of exercise training on skeletal muscle tissue regeneration**

To our knowledge, the present trial is the first one showing an increase in the number of capillaries containing vWF\textsuperscript{+} endothelial cells and CD34\textsuperscript{+} cells as a result of a training intervention in patients with CHF. It remains to be clarified, whether the CD34\textsuperscript{+} cells can be
associated to capillaries in the skeletal muscle support capillarization by paracrine effects, or whether they directly contribute to vessel formation by in situ-differentiation, or whether both mechanisms are involved. It is conceivable, that the training-induced increase in VEGF induces a pro-angiogenic milieu, supporting neovascularization. Additionally, the ability of CPCs to contribute to tissue vascularization and endothelial repair not only depends on CPC number but also on their functional capacity. However, both number and functional properties of CPCs were found to be significantly increased by the exercise training program in the present trial.

**Limitation of study**

**Change in VO$_2$max as primary study endpoint:** Improvement in physical work capacity after exercise training is well established in CHF patients. However, a NYHA III population has never been investigated in a prospective manner so far. Based on the results of one of our previous training trials with pre-dominantly NYHA II patients, we estimated a 20% less treatment effect with regard to VO$_2$max in NYHA III patients and used this value for the power calculation. However, this assumed improvement in VO$_2$max of +3.8 mL/min/kg based on our clinical experience and refered to theoretical considerations. Indeed this represents a large treatment effect, that could not be achieved in the present prospective, randomized trial.

**Measurement of peripheral vasomotion:** Since it is conceivable that interventions like e.g. exercise training influence the maximal flow stimulus, the change in FMD of a conduit artery may also be related to changes in flow, which is indirectly mediated by changes in the microcirculation rather than improvement of endothelial function of the conduit vessel per se. Flow velocity at rest and under peak hyperemia were not determined in the present study. Therefore, we cannot exclude the influence of flow on our results. This clearly represents a limitation of the method. However, in previously published studies with similar interventions, the
flow stimulus remained unchanged. We also cannot exclude differences within the reactive hyperemia stimulus per se in this advanced CHF population, since the previous observations relate to the findings in patients with pre-dominantly moderate CHF. Additionally, we did not determine endothelium-independent dilatation, a value indicative for smooth muscle cell function, possibly also affected in CHF patients. Previously published data from our group revealed that endothelium-independent vasodilatation after stimulation with nitroglycerine was not affected by exercise training and giving that the present patient population frequently showed excessive hypotensive response to the standard dose of 0.2 mg nitroglycerine, we considered it ethically not permissible to continue testing endothelium-independent vasodilation. Therefore, we cannot exclude also an improved responsiveness of the smooth muscle partially contributing to our FMD findings.
SOURCES OF FUNDING

This work was supported by a grant from the German Foundation for Heart Research (DSHF F21/02).

DISCLOSURES

None.
REFERENCES


Table 1: Clinical characteristics and medication at study begin

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<th>Control n=19</th>
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<td>---------------------------------------------</td>
<td>-----</td>
<td>-----</td>
<td>------</td>
</tr>
<tr>
<td>Aspirin or Clopidogrel or Cumarine [n]</td>
<td>17</td>
<td>17</td>
<td>1.000</td>
</tr>
<tr>
<td>Beta blocker [n]</td>
<td>17</td>
<td>19</td>
<td>0.486</td>
</tr>
<tr>
<td>ACE inhibitor or AT II blocker [n]</td>
<td>18</td>
<td>19</td>
<td>1.000</td>
</tr>
<tr>
<td>Aldosterone antagonist [n]</td>
<td>18</td>
<td>15</td>
<td>0.105</td>
</tr>
<tr>
<td>Other diuretic [n]</td>
<td>17</td>
<td>19</td>
<td>0.486</td>
</tr>
<tr>
<td>Statin [n]</td>
<td>14</td>
<td>13</td>
<td>0.714</td>
</tr>
<tr>
<td>Digitalis [n]</td>
<td>10</td>
<td>4</td>
<td>0.068</td>
</tr>
<tr>
<td>Allopurinol [n]</td>
<td>7</td>
<td>3</td>
<td>0.151</td>
</tr>
</tbody>
</table>

Data as mean±SD. Abbreviations: LV: left-ventricular; VO₂ max: maximal oxygen consumption; FMD: flow-mediated dilatation; SR: sinus rhythm; AF: atrial fibrillation; ICD: implantable cardioverter defibrillator; ACE inhibitor: angiotensin converting enzyme inhibitor; AT II blocker: angiotensin II subtype I receptor blocker.
### Table 2: Spiroergometry

<table>
<thead>
<tr>
<th></th>
<th>Training</th>
<th>Control</th>
<th>p value initial vs. 12 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>initial 12 weeks</td>
<td>initial 12 weeks</td>
<td></td>
</tr>
<tr>
<td>VO$_2$max [mL/min*kg]</td>
<td>15.3±3.3</td>
<td>17.8±3.2</td>
<td>15.4±3.8 14.7±3.7</td>
</tr>
<tr>
<td>VO$_2$VT [mL/min*kg]</td>
<td>11.9±3.6</td>
<td>14.2±2.7</td>
<td>11.8±3.5 12.0±3.3</td>
</tr>
<tr>
<td>VE / VCO$_2$ slope</td>
<td>37.1±7.6</td>
<td>33.7±6.4</td>
<td>38.8±11.3 38.1±11.3</td>
</tr>
<tr>
<td>Maximal Watt</td>
<td>78±15</td>
<td>94±23</td>
<td>76±23 69±26</td>
</tr>
<tr>
<td>Exercise duration [s]</td>
<td>525±122</td>
<td>638±156</td>
<td>471±179 425±190</td>
</tr>
<tr>
<td>HR at rest [bpm]</td>
<td>72±12</td>
<td>74±11</td>
<td>75±12 73±13</td>
</tr>
<tr>
<td>Sys BP at rest [mmHg]</td>
<td>111±15</td>
<td>112±17</td>
<td>117±16 112±17</td>
</tr>
<tr>
<td>Dia BP at rest [mmHg]</td>
<td>73±13</td>
<td>75±28</td>
<td>77±11 72±11</td>
</tr>
</tbody>
</table>

Data as mean±SD; Abbreviations: VO$_2$max: maximal oxygen consumption; VO$_2$VT: oxygen consumption at ventilatory threshold; HR: heart rate; Sys: systolic; Dia: diastolic; BP: blood pressure.
Table 3: Echocardiography

<table>
<thead>
<tr>
<th></th>
<th>Training</th>
<th>Control</th>
<th>p value initial vs. 12 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>initial 12 weeks</td>
<td>initial 12 weeks</td>
<td></td>
</tr>
<tr>
<td>LV-EF [%]</td>
<td>24.1±5.1 33.5±5.7</td>
<td>24.5±4.3 23.7±4.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LV-ESD [mm]</td>
<td>60.0±7.3 51.3±7.8</td>
<td>56.8±8.0 58.8±9.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LV-EDD [mm]</td>
<td>69.5±7.5 62.6±6.8</td>
<td>65.7±7.9 66.9±9.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FS [%]</td>
<td>13.7±2.6 18.4±5.0</td>
<td>13.8±4.1 12.3±3.3</td>
<td>0.003</td>
</tr>
<tr>
<td>LV-ESV [mL]</td>
<td>200.4±95.4 141.8±46.7</td>
<td>164.6±50.5 168.8±52.9</td>
<td>0.001</td>
</tr>
<tr>
<td>LV-EDV [mL]</td>
<td>266.7±104.8 208.6±54.7</td>
<td>221.1±63.0 222.8±65.9</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Data as mean±SD; Abbreviations: LV-EF: left ventricular ejection fraction; LV-ESD: left ventricular end-systolic diameter; LV-EDD: left ventricular end-diastolic diameter; FS: fractional shortening; LV-ESV: left ventricular end-systolic volume; LV-EDV: left ventricular end-diastolic volume.
FIGURE LEGENDS

Figure 1: Study flow diagram

In this study, 18 patients were randomized to 12 weeks of exercise training and 19 patients to the control group. One patient of each group withdraw consent of study participation during the follow up period of 12 weeks. Therefore, paired data are available in 17 patients of the training and the control group, respectively.

Figure 2: Peripheral endothelial function

A: Individual changes of radial artery flow-mediated dilatation (FMD) in the training group. P-value: * p<0.001 versus control. Data as mean±SD.

B: Individual changes of radial artery FMD in the control group. Data as mean±SD.

Figure 3: Number and function of CPC

A: Serum concentration of VEGF as determined by ELISA. Filled bars: study begin; open bars: 12 weeks follow-up. P-value: * p=0.02 versus control. Data as mean±SD.

B: Number of circulating CD34⁺ / KDR⁺ CPCs as determined by FACS. Filled bars: study begin; open bars: 12 weeks follow-up. P-value: * p=0.032 versus control. Data as mean±SD.

C: Migratory capacity of CPCs. Filled bars: study begin; open bars: 12 weeks follow-up. P-value: * p<0.001 versus control. Data as mean±SD.
Figure 4: Skeletal muscle neovascularization

A: Number of vWF⁺ capillaries/ fiber. Filled bars: study begin; open bars: 12 weeks follow-up. P-values: * p<0.001 versus control for the change. Data as mean±SD.

B: Number of CD34⁺ cells/ hpf in skeletal muscle as determined by immunohistochemistry. Filled bars: study begin; open bars: 12 weeks follow-up. P-values: * p<0.01 versus control for the change. Data as mean±SD.
Randomisation und initial measurements

37 patients

Training

n=18

1 drop out

Follow-up after 12 weeks completed

n=17

Control

n=19

1 drop out

1 death

n=17
Exercise Training in Patients with Advanced Chronic Heart Failure (NYHA IIIb) Promotes Restoration of Peripheral Vasomotor Function, Induction of Endogenous Regeneration, and Improvement of Left-Ventricular Function
Sandra Erbs, Robert Höllriegel, Axel Linke, Ephraim B. Beck, Volker Adams, Stephan Gielen, Sven Möbius-Winkler, Marcus Sandri, Nicolle Kränkel, Rainer Hambrecht and Gerhard Schuler

Circ Heart Fail. published online April 29, 2010;
Circulation: Heart Failure is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1941-3289. Online ISSN: 1941-3297

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SUPPLEMENTAL MATERIAL

Exercise training in patients with advanced chronic heart failure (NYHA IIIb) promotes restoration of peripheral vasomotor function, induction of endogenous regeneration, and improvement of left-ventricular function

Sandra Erbs*, M.D., Robert Höllriegel**, M.D., Axel Linke1, M.D., Ephraim B. Beck1, M.D., Volker Adams1, Ph.D., Stephan Gielen1, M.D., Sven Möbius-Winkler1, M.D., Marcus Sandri1, M.D., Nicolle Kränkel2, Ph.D., Rainer Hambrecht3, M.D., Gerhard Schuler1, M.D.

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SUPPLEMENTAL METHODS

Spiroergometry (exercise testing and respiratory variables)
After echocardiography, blood collection, skeletal muscle biopsy, and assessment of endothelial function, patients underwent symptom-limited ergospirometry. Exercise testing was performed on a calibrated, electronically braked bicycle in an upright position. Workload was increased progressively every 3 minutes in steps of 25 W beginning at 25 W. Respiratory gas exchange was determined continuously throughout the exercise test with determination of maximal oxygen uptake (VO$_{2\text{max}}$) and oxygen uptake at ventilatory threshold (VO$_{2\text{VT}}$). Ventilatory threshold was defined as the oxygen uptake before the systemic increase in the ventilatory equivalent for oxygen without a concomitant increase in the ventilatory equivalent for carbon dioxide. Ventilatory efficiency, as determined by the minute ventilation (VE)-carbon dioxide production (VCO$_2$) slope as a powerful prognostic marker was assessed.

Two-dimensional echocardiography
Before exercise testing a conventional transthoracic echocardiography was performed. Left-ventricular diameters and volumes were acquired as an average of 5 measurements according to guidelines for two-dimensional echocardiography. Left-ventricular ejection fraction was determined from left-ventricular volumes (as an average from five consecutive beats) - and not from diameters - as recommended in the guidelines. Furthermore, only one experienced investigator, who was blinded to patient identity, group assignment, and intervention status performed all echocardiographic analyses.
**Measurement of peripheral flow-mediated vasodilatation of radial artery (FMD)**

A high-resolution echo-tracking angiometer (NIUS 02, Asulab Research laboratory, Neuchatel, Switzerland) was used for non-invasive measurement of radial artery internal diameter.

FMD measurements were performed before exercise testing.

After a rest period of at least 20 minutes and under standardized conditions (quite, temperature-controlled room, fasting condition) baseline diameter was determined.

A 10 MHz transducer was positioned perpendicular to the radial artery about 5 cm proximal to the wrist, without direct skin contact. During measurements the patient was in supine position with the forearm resting on a special support device to avoid unintentional motions.

For determination of FMD, the brachial artery was occluded by inflating a blood pressure cuff to 50 mmHg above the systolic blood pressure for 5 minutes. The radial artery internal diameter was continuously recorded at least 180 seconds after cuff release, and the maximal diameter was recorded and related to baseline diameter to determine FMD.

Only one experienced investigator, who was blinded to patient identity, group assignment, and intervention status performed FMD measurements.

Also for the secondary endpoint FMD, a power calculation was performed. A difference in the treatment effects between the groups of 200 µm was assumed with an estimate of the standard deviation of 170 µm. With an a error of 0.05 and a b error of 0.10 (power 90%), minimal group size is estimated to be 17 patients in each group. The calculation of a treatment difference of 200 µm based on a previously published study from our group investigating femoral artery endothelial function in patients with CHF before and after training¹.
In that study, the endothelium-dependent increase in femoral artery diameter (at baseline 6 mm) in response to exercise training was about 0.5 mm. This value was indicative for a significant treatment effect. Given the fact, that radial artery size is appr. 40% of that from femoral artery, we assumed a 40% radial artery endothelial response according to 0.2 mm / 200 µm.

**Skeletal muscle biopsies**

Before maximal spiroergometry at baseline and again after 12 weeks percutaneous needle biopsies (approximately 50 mg/ biopsy) were obtained from the middle part of the vastus lateralis muscle under local anesthesia with 2% lidocaine solution using the method of Bergström. After 0.5 cm incision through skin and fascia lata, the needle was consistently inserted to a depth of 40 to 60 mm to cut the samples. The biopsies were either snap frozen in liquid nitrogen and stored at –80°C or fixed with 4% buffered formaldehyde before paraffin embedding.

**Collection of blood samples**

Venous blood samples were obtained before exercise testing at baseline and after 12 weeks to determine fasting lipid profile and the other routine laboratory parameters as well as the below mentioned parameters of regenerative capacity.

**Measurement of number and function of circulating progenitor cells**

*Quantification by flow cytometry*

At beginning and at 12 weeks, the number of CPCs was analyzed in venous blood by flow cytometry. To quantify the content of circulating EPCs by FACS analysis, a volume of 200 µL peripheral blood was incubated for 20 minutes with biotinylated
anti-human KDR (R&D Systems, Wiesbaden, Germany) and followed by the addition of anti-human CD34 (Miltenyi, Bergisch Gladbach, Germany). After incubation, the erythrocytes were lysed, and the remaining cells were washed with PBS and fixed in 2% paraformaldehyde before analysis using a FACS Calibur (Becton-Dickinson). To quantify the amount of KDR<sup>+</sup>CD34<sup>+</sup> double-positive cells, the mononuclear cell fraction was gated and analyzed for the expression of CD34 and CD3. Only the CD34<sup>+</sup>CD3<sup>−</sup> cells were finally investigated for the content of KDR<sup>+</sup>/CD34<sup>+</sup> double-positive cells. Mixing experiments revealed that cells with a frequency as low as 0.005% could be detected by FACS analysis.

**Isolation, cultivation, and characterization of EPCs used for functional analyses**

Mononuclear cells (MNC) were isolated by density gradient centrifugation from 20 mL of peripheral blood. After isolation 2x10<sup>6</sup> cells were plated on gelatin-coated 4-well chamber slides (NUNC, Wiesbaden, Germany) in medium 199 containing 20% fetal calf serum (Gibco), endothelial cell growth supplement (CCPro, Neustadt, Germany), and antibiotics (100 U penicillin/100 µg/mL streptomycin; Gibco). After 7 days in culture, non-adherent cells were removed by a thorough rinsing with cell culture medium, and adherent cells were characterized by cytochemical methods. To investigate the phenotype of the attached cells by FACS analysis, the cells were harvested after 7 days in culture by PBS/EDTA and stained with anti-VE-cadherin-FITC (Bender Medsystems), anti-KDR-biotin (R&D Systems) followed by streptavidin-PE (Caltac), and anti-CD3-PerCP (Becton Dickinson) before being analyzed by laser scanning cytometry (LSC) (CompuCyte).

**Measurement of functional capacity of CPCs by migration assay**

The migratory capacity of CPCs was assessed using an invasion chamber assay (BD Biosciences; San Jose, Calif). Isolated CPCs were detached using 1 mmol/L EDTA in
PBS (pH 7.4), harvested by centrifugation, resuspended in 500 µL of EBM, conted, and placed in the upper chamber of a modified Boyden chamber. The chamber was placed in a 24-well culture dish containing EBM and human recombinant SDF-1. After 24 hours of incubation at 37° C, the lower side of the filter was washed with PBS and fixed with 2% paraformaldehyde. For quantification, cell nuclei were stained with 4’,6-diamidino-phenylidole. Migrated cells into the lower chamber were counted manually in 3 random microscopic fields (Lit).

Measurement of circulating growth/ homing factors, systemic markers of inflammation and oxidative stress

Plasma levels of vascular endothelial growth factor (VEGF), stromal-derived factor 1 (SDF-1), tumor necrosis factor alpha (TNFalpha), and lipid peroxides (LPO) were measured by highly sensitive ELISA (VEGF, SDF-1, TNFalpha: R&D-Systems, Wiesbaden, Germany; lipid peroxides: Immundiagnostik, Bensheim, Germany). according to the manufacturers instructions. Results were compared with standard curves, and the lower detection limits were: VEGF, 9 pg/mL; SDF-1, < 47 pg/mL; TNFalpha, 0.18 pg/mL; LPO, 7 µmol/L. The intra-assay and interassay variability was <10%. Measurements were performed in duplicate.

Measurement of neovascularization of skeletal muscle

Sections of 2 µm were cut throughout the paraffine embedded tissue block. Identification of CD34+ cells in the skeletal muscle: CD34+ cells were identified in paraffin sections of skeletal muscle by immunohistochemistry using as specific monoclonal antibody (Dako, Hamburg, Germany). The density of premature
capillaries containing CD34$^+$ cells in the skeletal muscle is expressed as number of CD34$^+$ /high power field (hpf) via light microscopy.

*Identification of von Willebrand factor (vWF$^+$) capillaries:* Additionally, mature capillaries were identified in paraffin sections of skeletal muscle by an anti-human vWF antibody (Dako, Hamburg, Germany). The density of mature capillaries was calculated as the number of vWF$^+$ vascular structures per skeletal muscle fiber.

**SUPPLEMENTAL REFERENCES**