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Randomized controlled trial using bosentan to enhance the impact of exercise training in subjects with type 2 diabetes mellitus

RANDOMISED CONTROLLED TRIAL USING BOSENTAN TO ENHANCE THE IMPACT OF EXERCISE TRAINING IN SUBJECTS WITH TYPE 2 DIABETES MELLITUS

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ABSTRACT

In type 2 diabetes patients, endothelin(ET)-receptor-blockade may enhance blood flow responses to exercise training. The combination of exercise training and ET-receptor-blockade may represent a more potent stimulus to improve vascular function, physical fitness, and glucose homeostasis. We assessed the effect of an 8-week exercise training program combined with either ET-blockade or placebo on vasculature, fitness and glucose homeostasis in people with type 2 diabetes. In a double blind randomized controlled trial, brachial endothelium-dependent and –independent dilation (using flow-mediated dilation (FMD) and glyceryl trinitrate (GTN), respectively), glucose homeostasis (using HOMA-IR), and physical fitness (maximal cycling test) were assessed in 18 men with type 2 diabetes (60±6 years). Subjects underwent an 8-week exercise training program with half of the subjects receiving ET-receptor-blockade (Bosentan) and the other half a placebo, followed by reassessment of the tests above. Exercise training improved physical fitness to a similar extent in both groups, but we did not detect changes in glucose homeostasis or vascular function in either group. This study suggests no adaptation in brachial and femoral artery endothelial function and glucose homeostasis after 8 weeks of training in type 2 diabetes patients. ET-blockade combined with exercise training does not additionally alter conduit artery endothelial function, physical fitness or glucose homeostasis in type 2 diabetes.

ClinicalTrials.gov identifier: NCT01779609

KEYWORDS: cardiovascular risk; exercise training; metabolic disease; shear stress; bosentan

INTRODUCTION

Type 2 diabetes mellitus is a rapidly growing problem in Western society. Cardiovascular disease and vascular complications represent the leading cause of morbidity and mortality in type 2 diabetes. Previous studies found an impaired endothelial function in patients with type 2 diabetes (McVeigh *et al.*, 1992; Clarkson *et al.*, 1996; Williams *et al.*, 1996), which is believed to contribute to the pathogenesis of cardiovascular disease in type 2 diabetes patients (Beckman *et al.*, 2002; Green *et al.*, 2004). Exercise training represents an effective strategy to improve endothelial function in conduit and resistance vessels and, consequently, reduce the risk of cardiovascular disease in symptomatic and asymptomatic subjects (see reviews) (Green *et al.*; Thijssen *et al.*).

Previous studies in animals (Langille & O'Donnell, 1986; Tuttle *et al.*, 2001) and humans (Green, 2009) demonstrated that repeated elevation in blood flow, and consequently shear stress (Thijssen *et al.*, 2009), is a key stimulus for vessels to adapt in function and structure (Tinken *et al.*, 2010). More specifically, recent studies demonstrated that the beneficial effects of exercise training on the vasculature largely depend on acute exercise-induced elevations in blood flow (Johnson *et al.*; Zhu *et al.*; Harris *et al.*, 2007, 2008; Tinken *et al.*, 2010). A previous study found that type 2 diabetes patients show an attenuated blood flow response to exercise compared to healthy controls (Lalande *et al.*, 2008). This diminished blood flow and shear stress response to exercise may prevent an optimal stimulus for adaptation of the vasculature in type 2 diabetes (Bauer TA, 2007; Lalande *et al.*, 2008), and may even contribute to microvascular complications (Womack *et al.*, 2009). Therefore, exercise training combined with pharmacological up- or downregulation of vasoactive substances may potentially lead to superior effects on the vasculature than exercise training alone (Buford *et al.*).

We recently demonstrated that systemic ET-1 blockade leads to an increased exercise-induced blood flow response during handgrip exercise in type 2 diabetes, but not in healthy controls (Schreuder *et al.*, 2014). Accordingly, exercise training combined with an ET-antagonist in type 2 diabetes patients may enhance the blood flow response and thereby the impact of exercise training on the vasculature and physical fitness. An increased blood flow has been demonstrated to represent a stimulus to upregulate glucose homeostasis, possibly via potentiation of insulin-driven capillary recruitment (Rattigan *et al.*, 2001; Clark *et al.*, 2003; De Filippis *et al.*, 2006; Lteif *et al.*, 2007). Therefore, the purpose of this study was to perform a randomised controlled trial (RCT) to assess the effect of an 8-week exercise-training program with either ET-blockade or a placebo on the vasculature, fitness and glucose homeostasis in patients with type 2 diabetes.

METHODS

Subjects

18 men (60±6 years) who were diagnosed with type 2 diabetes for at least two years were recruited from the general population through advertisements for our double blind randomized controlled trial (RCT; ClinicalTrials.gov identifier: NCT01779609). Exclusion criteria included overt cardiovascular disease, smoking, type I diabetes mellitus, age <40 or >65, diabetes-related manifest vascular complications, increased levels of liver aminotransferases (ASAT >120 U/L ALAT >135 U/L), glibenclamide use, use of calcineurin inhibitors, use of HIV-drugs due to a possible interference with Bosentan, and use of drugs that interfere with CYP3A4, CYP2C9, CYP3A4 and/or CYP2C9. The study procedures were approved by the medical ethical committee of the Radboud University Nijmegen Medical Centre and adhered to the Declaration of Helsinki. All subjects gave written informed consent before participation in this study. During this study, no adverse events occurred in the study population.

Experimental design

Patients reported to the laboratory for initial assessment of brachial artery function and structure, glucose homeostasis, and physical fitness. Subsequently, all subjects underwent an 8-week exercise-training program. In a randomized, double-blind manner, subjects combined exercise training with either a dual ET-blocker (EX+ET-blocker) or a placebo (EX+placebo) for 8 weeks. Ingestion of the dual ET-blocker (Tracleer, Actelion Pharmaceuticals) during the first 4 weeks represented 62.5 mg twice per day, followed by four weeks of 125 mg twice daily. This dosage has been used in previous studies and has proven to be effective and well tolerated (Monfredi *et al.*, 2011). The other group used a placebo that had the same size, weight, taste, and colour as the dual ET-blocker and was also taken twice daily. Subjects and

researchers were blinded for the ingestion of placebo or the ET-blocker. Randomization was executed by the Radboud University Nijmegen Medical Centre pharmacy, who provided sequentially numbered containers. Immediately after the 8-week intervention, within seven days of the last exercise bout, we repeated all tests as listed above.

Measurements: physical fitness and subject characteristics

Subject characteristics. During a screening procedure at the first visit all subjects completed a questionnaire concerning their medical history and medication use. We measured height, weight, resting blood pressure after a 5-minute seated rest using a manual sphygmomanometer. A venous blood sample was taken for assessment of fasting glucose, insulin, total cholesterol, HDL, LDL, triglycerides, ASAT and ALAT. From the glucose and insulin levels we calculated the HOMA-IR index as a valid measure of insulin resistance (Matthews *et al.*, 1985).

Physical fitness. On a subsequent day, subjects performed an incremental cycle exercise test to examine maximal workload and peak oxygen consumption. Also, data from this test were used to determine maximal heart rate, which was used to calculate work-load during exercise training. Each subject performed an incremental maximal exercise test on a cycling ergometer (Lode, Excalibur, Groningen, the Netherlands) before and after the training program. These tests started at a power output of 10 W for 1 min and power output increased by 10 W/min until exhaustion. Subjects were instructed to maintain a cadence of between 60 and 80 rpm during the test. We continuously recorded oxygen consumption (VO_2, in ml O_2/kg per min), ventilation (Ve, in l/min), respiratory quotient (RQ) (Oxycon IV, Jaeger, Germany) and heart rate (HR, in bpm). Furthermore, we measured blood lactate levels (mmol/l) using Accutrend® Lactate (Roche Diagnostics GmbH, type 3012522, Mannheim, Germany) before and 2 min after finishing the maximal exercise test.

Measurements: vascular function

All subjects refrained from alcohol, caffeine, and vigorous physical exercise for at least 24 hours prior to testing, and were fasted for at least 6 hours. All tests were performed in laboratory conditions with constant temperature (20°C) and humidity (35%). We performed all tests between 8 AM and 4 PM, but to control for variation in FMD pre- and post exercise training measurements for each subject were performed at the same time of day to prevent diurnal variation in the FMD response (Jones *et al.*).

Brachial artery endothelium-dependent dilation. Measurement of brachial artery endothelium-dependent dilation (using flow-mediated dilation (FMD)) was performed by an experienced vascular sonographer. A 10 MHz multifrequency linear array probe attached to a high resolution ultrasound machine (T3000; Terason, Burlington, MA, USA) was used to image the brachial artery in the distal one-third of the upper arm. When an optimal image was obtained, the probe was held stable and the ultrasound parameters were set to optimize the longitudinal, B-mode images of lumen–arterial wall interface.

For assessment of the pre-exercise FMD, subjects rested in the supine position for a period of at least 15 minutes to facilitate baseline assessment of artery diameter and blood flow. To examine brachial artery FMD, the arm was extended and positioned at an angle of ~80 degrees from the torso. A rapid inflation and deflation pneumatic cuff was positioned on the forearm of the imaged arm immediately distal to the olecranon process to provide a stimulus of forearm ischaemia. Continuous Doppler velocity assessment was obtained using the lowest possible insonation angle (always <60 degrees), which did not vary during each measurement. The forearm cuff was inflated to 220 mmHg for 5 min. Diameter and flow recordings resumed 30 s prior to cuff deflation and continued for 3 min thereafter. Assessment

of brachial artery FMD was repeated after the exercise bout and started within 2 min of cessation of exercise. Time to peak was calculated from the point of cuff deflation to the maximum postdeflation diameter. Calculation of FMD and time to peak were therefore observer-independent and based on standardized algorithms applied to data, which had undergone automated edge-detection and wall-tracking.

Brachial artery endothelium-independent dilation. Following a rest period of at least 15 minutes to allow brachial artery diameter and flow to return to baseline levels, a 1-min baseline recording of diameter and flow was taken. Subsequently, brachial artery endothelium-independent vasodilation was examined after administration of a single spray of sublingual GTN (400 µg), an NO donor. This was followed by 10 min continuous recording of brachial artery diameter and blood flow.

Brachial artery peak blood flow. After a >15-min rest period, a 1 min baseline recording of brachial artery diameter and blood flow was performed. Next, brachial artery dilation was examined after a 5-min period of ischemia, consisting of 1 min ischemia, followed by 3-min isotonic handgrip exercise and a final 1 min of ischemia. Handgrip exercise involved 1 contraction every 2 s of a 3-kg load. Peak blood flow was defined as the blood flow area under the curve of the highest 10s window after cuff deflation. The peak hyperemic forearm blood flow in response to this stimulus in humans provides a valid and accepted index of resistance artery size or remodeling (Naylor *et al.*, 2005). We resumed diameter and flow recordings 30 s prior to cuff deflation and continued for 3 min thereafter.

Superficial femoral artery endothelium-dependent dilation. After measurement of the brachial artery, we continued with measurements of the superficial femoral artery. The Terason ultrasound equipment was used to measure femoral artery diameter and flow. The cuff was placed at the thigh of the imaged leg, directly above the knee. Measurement of superficial femoral artery endothelium-dependent function was performed as described above for the

brachial artery. Measurements continued for 5 minutes after cuff deflation. A previous study found that this procedure results in a largely NO-mediated, endothelium-dependent dilation of the superficial femoral artery (Thijssen *et al.*).

Exercise training

Exercise training was performed over an 8-week training period with subjects visiting our facility 3 times a week. Each exercise session was supervised by a well-trained researcher and consisted of a 5-minute warming-up, followed by a circuit of resistance exercises (leg press, calf raise, leg curl, leg extension, lower back, abdominal crunch, 3 series of 12 repetitions each, with 1 minute of rest between sets within each exercise) interspersed with aerobic activities (e.g. cycling, running) (Maiorana *et al.*, 2002; Watts *et al.*, 2004). The total protocol was as follows: 5 minutes warming-up (cycling), 5 minutes of cycling, leg curl, leg extension, 5 minutes of running, lower back, abdominal crunch, 5 minutes of cycling, leg press, calf raise, 5 minutes of running. The total duration of a training session was approximately 60 minutes. A heart rate monitor (Polar Electro Oy, Kempele, Finland) was used to continuously monitor heart rate during the aerobic exercise and heart rate was maintained at 70-75% of heart rate reserve. The intensity level for each of the resistance exercises was set at a level which enables the participant to complete the three series of 12 repetitions. Intensity was increased each week under the guidance of the trainers. If a participant missed a supervised exercise session, an extra session was planned in the same or following week, so that each participant performed a total of 24 supervised sessions (100% compliance).

Data Analysis

Post-test analysis of brachial artery diameter and velocity was performed using custom-designed edge-detection and wall-tracking software, which is independent of investigator bias

(Woodman *et al.*, 2001). Briefly, the echo-Doppler signal was real-time encoded and stored as a digital file. Subsequent software analysis of these data was performed at 30 Hz using an icon-based graphic programming language and toolkit (LabView 6.02; National Instruments, Austin, TX). The program allows users to identify a region of interest on the clearest portion of the vascular wall. It then identifies, via the intensity of the brightness of the walls versus the lumen of the vessel, the walls of the artery. B-mode image were viewed, and regions of interest were selected for diameter and blood velocity. From this synchronized diameter and velocity data, blood flow (the product of lumen cross-sectional area and Doppler velocity) was calculated at 30 Hz. Baseline diameter, flow, and shear rate were calculated as the mean of data acquired across the 1 minute preceding the cuff inflation period.

FMD was calculated using the baseline and peak diameter following cuff deflation. Peak diameter was automatically detected according to an algorithm, which identified the maximum bracket of data subsequent to performance of a moving window smoothing function. This smoothing routine calculates the median value from 100 consecutive samples, before the window shifts to the next bracket of data, which shares 20% overlap with the preceding bracket. The maximum value of all the calculated median values is then automatically detected and chosen to represent the peak of the post-deflation artery diameter curve. FMD was calculated as the percentage rise of this peak diameter from the preceding baseline diameter. We have shown that reproducibility of diameter measurements using this semi-automated software is significantly better than manual methods, reduces observer error significantly, and possesses an intra-observer coefficient of variance of 6.7% (Woodman *et al.*, 2001). Analysis of the conduit artery dilator capacity and glyceryl trinitrate measurements was performed using the same methods. Shear rate is represented as shear rate area-under-the-curve between the moment the cuff is released and the end of the FMD measurement (3 min for brachial, 5 min for superficial femoral artery).

Statistical analysis

Statistical analyses were performed using SPSS 20.0 (SPSS, Chicago (Illinois), USA) software. According to Woodman *et al.* our sample size is sufficient to detect clinically relevant differences in our primary outcome measures (Woodman *et al.*, 2001). All data are reported as mean (SD) unless stated otherwise, and statistical significance was set a priori at $P \leq 0.05$. Unpaired Student's *t*-tests were used to compare baseline values between groups. A two-way repeated measures ANOVA was used to examine changes in physical fitness, glucose homeostasis and vascular function/structure across the exercise-training period ('training'; 0 *versus* 8 weeks), and whether the magnitude of exercise training mediated adaptations differ between groups ('group'; EX+ET-blockade *versus* EX+placebo). When a significant main- or interaction-effect was found, post-hoc comparisons were performed to identify differences with/without ET-receptor blockade. Post-hoc analysis was performed using the least significant difference (LSD) method for pair-wise multiple comparisons when a significant main effect was observed (Perneger, 1998). According to a recent study by Atkinson *et al.*, inadequate scaling for FMD would be present if the upper confidence limit of the regression slope of the relationship between logarithmically transformed base diameter and peak diameter is less than one (Atkinson *et al.*, 2013). In such an event, FMD% is not an appropriate measure to estimate endothelial function. We checked our data for this phenomenon, and where appropriate we performed the allometric modelling solution proposed by Atkinson *et al.*(Atkinson *et al.*, 2013).

RESULTS

Baseline characteristics

Prior to the 8-week intervention, we found no significant differences between EX+ET-

blockade (N=8) and EX+placebo (N=10) for height, weight, BMI, blood pressure, total cholesterol, lipid profile, and plasma-Endothelin-1 (all comparisons P>0.05, Table 1). The 8-week exercise training intervention did not alter these parameters in EX+ET-blockade nor in EX+placebo, except for a significant decrease in triglycerides in both groups (Table 1).

Physical fitness

Prior to the intervention, we found significantly lower maximal workload in EX+ET-blockade compared to EX+placebo, but no differences in maximal oxygen uptake (Table 2). Exercise training resulted in a significant increase in maximal oxygen uptake and maximal load, which was comparable between both groups (Table 2). For both groups, maximal heart rate and peak lactate were similar before and after the exercise training program (Table 2).

Glucose homeostasis

Before training, we found no differences in insulin, glucose and HOMA-IR between EX+ET-blockade and EX+placebo groups (all comparisons P>0.05, Table 3). In both groups, we found no main or training*group-interaction effect on blood glucose and insulin levels, and insulin resistance (HOMA-IR) (Table 3).

Vascular function

At baseline, we found no differences between groups in brachial artery FMD, peak diameter, peak blood flow and GTN or superficial femoral artery FMD (all comparison P>0.05, Table 4).

Brachial artery endothelium-dependent dilation. Exercise training with or without ET-receptor blockade did not change brachial artery flow mediated dilation, baseline diameter or shear rate area-under-the-curve (Table 4). We found a significant training*group interaction

for shear rate area-under-the-curve (P=0.009, table 4). Post-hoc testing revealed that the exercise training program significantly decreased shear rate area-under-the-curve in the EX+ET-blockade group (P=0.006), but not in the EX+placebo group (P=0.363).

Brachial artery endothelium-independent dilation. Endothelium-independent vascular function, measured as the dilatory response to a single sublingual dose of glyceryl trinitrate (GTN), was not influenced by exercise training with or without ET-receptor blockade (Table 4). We also found no impact of exercise training and/or ET-blockade on FMD/GTN-ratio (Table 4).

Brachial artery peak blood flow. We found a significant training*group interaction for the impact of the 8-week intervention on brachial artery peak blood flow. Post-hoc testing revealed that the exercise training program significantly increased peak blood flow in the EX+ET-blockade group (P=0.050), but not in the EX+placebo group (P=0.086) (Table 4).

Superficial femoral artery endothelium-dependent dilation. In the superficial femoral artery, exercise training with or without ET-receptor blockade did not change flow mediated dilation, baseline diameter or shear rate area-under-the-curve (Table 4).

DISCUSSION

As a logical follow-up of a recent study where we found ET-blockade to enhance exercise-induced blood flow in type 2 diabetes (Schreuder *et al.*, 2014), the purpose of this study was to perform a randomised controlled trial (RCT) to assess the effect of an 8-week exercise-training program combined with ET-blockade. First, we confirmed the potent effect of exercise training given the significantly improved peak oxygen uptake (P=0.006) and peak workload (P=0.001). In contrast to our hypothesis, we found no effect of exercise training on glucose homeostasis or vascular function in the upper or lower limbs. More importantly, we found that ET-blockade did not have any superior effects on the impact of exercise training on

our primary outcome parameters. This suggests that ET-blockade, despite the acute effects on blood flow, does not potentiate the effect of exercise training on the vasculature, fitness and glucose homeostasis in type 2 diabetes.

Exercise training significantly increased peak oxygen consumption with 12%, indicating that our training intervention was successful in improving physical fitness levels. The increase in peak oxygen consumption relates to training itself, since we did not find any differences in peak heart rate and lactate between both incremental cycling tests. Despite these training effects, we found no effect of our 8-week intervention on glucose homeostasis. Several other studies that investigated the effect of an 8-26 weeks training exercise program have found that exercise training improves glucose homeostasis (Boule *et al.*, 2001; Maiorana *et al.*, 2002; Snowling & Hopkins, 2006), whereas other studies with similar duration and intensity do not confirm this finding (Kaplan *et al.*, 1987; Lehmann *et al.*, 1995; Tessier *et al.*, 2000). A potential explanation for this disparity in the literature is that glucose homeostasis in older type 2 diabetes patients (>55 years) is less likely to improve during exercise training compared to younger subjects (Zierath & Wallberg-Henriksson, 1992). An alternative explanation is that *a priori* levels of glucose influence the ability of exercise training to improve glucose homeostasis. Indeed, Maiorana *et al.* found an improvement in fasting glucose from 12.0±0.5 to 9.8±0.5 mmol/L (Maiorana *et al.*, 2002), which both represent relatively high resting levels of glucose. In contrast, we found pre-training fasting glucose levels that were well below the post-training values reported by Maiorana et al. When comparing responders (n=6) and non-responders (n=12) in our study regarding the impact of exercise training on glucose levels, we did not find differences between groups for baseline characteristics or medication use. Therefore, it is unlikely that differences in baseline characteristics or medication use influenced our outcome parameters.

In the present study, we did not find any effect of adding ET-blockade to exercise training on circulating resting insulin or insulin sensitivity. Animal studies have suggested that ET-1 stimulates insulin release in pancreatic islets (Gregersen *et al.*, 1996; De Carlo *et al.*, 2000; Lai *et al.*, 2007). However, ET-1 has also been shown to reduce pancreatic blood flow (Takaori *et al.*, 1992; Liu *et al.*, 1995), which could interfere with normal pancreatic function. ET_A-blockade may reverse this decreased flow, but literature is not unequivocal on this point (Foitzik *et al.*, 1998; Lai *et al.*, 2007). Nonetheless, in our study we observed no impact of 8 weeks Bosentan ingestion on insulin levels or insulin resistance.

We found no effect of exercise training on flow-mediated dilation in the brachial or superficial femoral artery. Given the number of subjects (total n=18), the fact that both arteries show similar responses, the double-blind nature of our study (including the analysis), and the small coefficient of variance that is achieved by our FMD-technique, we believe it is unlikely that measurement or methodological errors explain our effect. A potential explanation for our findings may relate to a time-course in vascular adaptations during exercise training in type 2 diabetes. A previous study found that changes in flow mediated dilation occur in the first two weeks of exercise, followed by a normalisation when exercise was continued in healthy men (Tinken *et al.*, 2008). If subjects in our study demonstrate a similar time-course, the post-exercise FMD after 8 weeks of training missed any adaptation that occurred in the FMD in response to exercise training. However, no previous study examined the potential presence of a time-course in adaptation in FMD in response to exercise training in subjects with cardiovascular risk or disease, who typically demonstrate *a priori* endothelial dysfunction. Alternatively, it is possible that exercise training in subjects with *a priori* endothelial dysfunction takes longer than normal to induce significant improvements in endothelial function. Either way, future studies should perform multiple assessments of FMD to examine

the time-course and/or adopt a longer exercise training duration.

Although changes in endothelial function after exercise training are frequently reported (Maiorana *et al.*, 2001; Colberg *et al.*, 2002), not all studies have consistently found this effect in type 2 diabetes (Colberg *et al.*, 2005; Sonne *et al.*, 2007). One explanation for the conflicting results relates to the baseline FMD, with a higher pre-training FMD allowing for less adaptation after exercise training. For instance, Maiorana *et al* found a significant increase in brachial artery FMD from 1.7±0.5% to 5.0±0.4%, where in our study, the mean baseline FMD in the EX+ET-blockade and EX+placebo groups was 4.3±1.2 and 3.6±2.3, respectively. An alternative explanation for our unexpected observation of a preserved FMD response after training is that shear rate during the FMD measurement was significantly lower in the EX+ET-blockade group. As shear is the main stimulus for flow mediated dilation (Thijssen *et al.*), this finding may indicate that the sensitivity of the endothelium was increased in the EX+ET-blockade group.

Previous studies in animals (Langille *et al.*, 1989) and humans (Green *et al.*; Tinken *et al.*, 2010; Naylor *et al.*, 2011; Birk *et al.*, 2012) have demonstrated that repeated increases in shear rate represent a key stimulus for vascular adaptation. In an earlier study we have shown that Bosentan acutely increases exercise-induced blood flow, but not shear rate. This may explain why ET-blockade did not result in a superior effect on changing endothelial function in the present study.

Limitations. Strong points of our study include a randomised, double-blind design, the use of semi-automated software, and the use of a highly controlled exercise protocol. Some limitations need to be addressed. First, we used a dual ET-1 blocker. Previous studies have

demonstrated that type 2 diabetes is specifically associated with an upregulation of the ET_A-, but not ET_B-receptors. Even though in a previous study we have successfully increased exercise-induced blood flow during a single bout of exercise using a dual ET-1 blocker (Schreuder *et al.*, 2014), larger effects may be achieved with selective ET_A-receptor blockade (Sachidanandam *et al.*, 2008; Settergren *et al.*, 2008). A second limitation is that our main comparison was between, as opposed to within subjects. A randomised cross-over design, with a sufficient 'wash-out and detraining' period between training periods would yield smaller variations and possibly larger effects. However, a major concern would be that subjects are truly untrained at baseline, and any feasible detraining period would not be comparable to the inactive period prior to the start of the study. Therefore, we believe that our set-up utilising between-subjects comparisons is the best practical option. Finally, we have powered our sample size to detect changes in endothelial function. Therefore, our sample size may be too small to detect changes in insulin and glucose levels, potentially explaining why we did not find an effect of training or Bosentan on glucose homeostasis.

In conclusion, 8 weeks of exercise training significantly improved physical fitness in patients with type 2 diabetes mellitus, but we did not detect any effect on glucose homeostasis or brachial and femoral artery endothelial function. Furthermore, in contrast to our hypothesis, ET-blockade had no additional effect on our primary outcome parameters. This suggests that ET-blockade does not potentiate the effect of exercise training on conduit artery endothelial function, fitness and glucose homeostasis in type 2 diabetes.

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CONTRIBUTION STATEMENT

THAS, MTEH and DHJT designed the study. THAS collected and analysed the data. THAS, DJD, MTEH and DHJT interpreted the data. THAS, DJD, MTEH and DHJT wrote the manuscript. DHJT had primary responsibility for final content. DHJT is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors read and approved the final manuscript. No conflicts of interest exist.

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Table 1. Body characteristics in patients with type 2 diabetes mellitus that used bosentan (EX+ET), and patients with type 2 diabetes mellitus that used placebo (EX+placebo). Data is presented as mean ± SD. P-values represent a two-way repeated measures ANOVA.

	EX+ET-blockade N=8		**EX+placebo N=10**		**2-way ANOVA**		
Parameter	*Pre*	*Post*	*Pre*	*Post*	Training	Group	Training*Group
Age (yrs)	60±5		59±6			0.611	
Height (cm)	174±8		178±4			0.185	
Weight (kg)	99.5±23.9	98.1±22.8	101.5±16.3	102.1±17.1	0.582	0.750	0.177
Body mass index (kg/m^2)	32.8±8.3	32.4±8.2	31.9±4.6	32.1±4.9	0.644	0.843	0.199
Systolic blood pressure (mmHg)	136±10	132±11	138±17	135±12	0.227	0.841	0.641
Diastolic blood pressure (mmHg)	83±9	78±5	86±11	83±8	0.079	0.727	0.239
Cholesterol (mmol/L)	4.7±0.9	4.4±0.7	4.0±1.1	3.8±0.9	0.133	0.138	0.815
High-density lipoprotein (mmol/L)	1.0±0.5	1.0±0.3	1.0±0.2	1.0±0.2	0.480	0.926	0.808
Low-density lipoprotein (mmol/L)	2.9±0.6	2.8±0.6	2.2±1.1	2.2±1.1	0.561	0.172	0.998
Triglycerids (mmol/L)	1.8±0.6	1.4±0.5	1.6±0.9	1.4±1.0	**0.011**	0.790	0.395
ET-1 (pg/mL)	1.0±0.6	0.9±0.6	0.5±0.4	0.7±0.6	0.856	0.095	0.247

Table 2. Outcome parameters pre and post 8-weeks of exercise training in patients with type 2 diabetes mellitus with either ET-blockade (EX+ET) or placebo (EX+placebo). Data is presented as mean ± SD. P-values represent a two-way repeated measures ANOVA.

	EX+ET-blockade (N=8)		**EX+placebo (N=10)**		**2-way ANOVA**		
Parameter	*Pre*	*Post*	*Pre*	*Post*	Training	Group	Training*Group
Peak Oxygen Uptake (mLO_2/min/kg)	20.7±1.6	23.8±4.2*	23.3±6.4	25.6±6.0*	**0.006**	0.339	0.619
Peak workload (Watt)	137±29	173±40*	171±34$^{\#}$	205±34*	**0.001**	**0.048**	0.834
Maximal Heart Rate (1/min)	159±11	159±10	156±12	159±12	0.467	0.788	0.371
Peak lactate (mmol/L)	7.1±3.8	8.6±1.0	8.4±2.0	9.8±2.7	0.284	0.447	0.932

*Post-hoc significantly different from Pre at P≤0.05, $^{\#}$Significantly different between groups at baseline at P≤0.05

Table 3. Glucose homeostasis outcome parameters pre and post an 8-week training intervention in patients with type 2 diabetes mellitus with either ET-blockade (EX+ET) or placebo (EX-placebo). Data is presented as mean ± SD. P-values represent a two-way repeated measures ANOVA.

	EX+ET-blockade (N=8)		**EX+placebo (N=10)**		**2-way ANOVA**		
Parameter	*Pre*	*Post*	*Pre*	*Post*	Training	Group	Training*Group
Glucose (mmol/L)	6.7±1.1	6.9±0.5	7.6±2.5	6.4±1.5	0.454	0.696	0.239
Insulin (mU/L)	10.8±5.3	11.0±4.4	15.4±9.4	17.0±13.0	0.582	0.214	0.666
HOMA-IR (10/%S)	3.2±1.4	3.4±1.5	5.4±3.8	5.1±4.5	0.990	0.176	0.700

Table 4. Vascular outcome parameters pre and post 8-weeks of exercise training in patients with type 2 diabetes mellitus with either ET-blockade (EX+ET) or placebo (EX-placebo). Data is presented as mean ± SD. P-values represent a two-way repeated measures ANOVA.

	EX+ET-blockade (N=8)		**EX+placebo (N=10)**		**2-way ANOVA**		
Brachial artery	*Pre*	*Post*	*Pre*	*Post*	Training	Group	Training*Group
Diameter (mm)	4.3±0.6	4.4±0.6	4.7±0.6	4.5±0.6	0.667	0.366	0.316
Flow Mediated Dilation (FMD, %)	4.3±1.2	4.8±2.5	3.6±2.3	4.1±1.8	0.335	0.413	0.982
Shear Rate$_{AUC}$ (s, 10^3)	22.6±8.9	15.5±7.0*	14.7±6.6	17.0±6.5	0.151	0.305	**0.009**
Glyceryl Trinitrate (GTN, %)	17.8±7.8	14.1±4.8	14.8±5.8	12.3±7.1	0.119	0.336	0.742
FMD/GTN	0.30±0.17	0.37±0.23	0.27±0.16	0.48±0.36	0.110	0.679	0.392
Peak blood flow$_{AUC}$ (mL/min)	603±246	854±293*	980±462	787±294	0.701	0.299	**0.008**
Superficial femoral artery							
Diameter (mm)	7.6±1.4	7.3±1.5	7.3±0.7	7.2±0.8	0.356	0.727	0.390
FMD (%)	3.1±0.9	3.1±1.9	3.2±1.3	3.1±1.5	0.960	0.910	0.841
Shear Rate$_{AUC}$ (s, 10^3)	4.2±2.7	5.3±4.5	4.8±3.8	7.0±5.2	0.160	0.523	0.641

*Post-hoc significantly different from Pre at P≤0.05