

Endurance training and detraining in mitochondrial myopathies due to single large-scale mtDNA deletions

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At present there are limited therapeutic interventions for patients with mitochondrial myopathies. Exercise training has been suggested as an approach to improve physical capacity and quality of life but it is uncertain whether it offers a safe and effective treatment for patients with heteroplasmic mitochondrial DNA (mtDNA) mutations. The objectives of this study were to assess the effects of exercise training and detraining in eight patients with single, large-scale mtDNA deletions to determine: (i) the efficacy and safety of endurance training (14 weeks) in this patient population; (ii) to determine the effect of more prolonged (total of 28 weeks) exercise training upon muscle and cardiovascular function and (iii) to evaluate the effect of discontinued training (14 weeks) upon muscle and cardiovascular function. Our results show that: (i) 14 weeks of exercise training significantly improved tolerance of submaximal exercise and peak capacity for work, oxygen utilization and skeletal muscle oxygen extraction with no change in the level of deleted mtDNA; (ii) continued training for an additional 14 weeks maintained these beneficial adaptations; (iii) the cessation of training (detraining) resulted in loss of physiological adaptation to baseline capacity with no overall change in mutation load. Patients' self assessment of quality of life as measured by the SF-36 questionnaire improved with training and declined with detraining. Whilst our findings of beneficial effects of training on physiological outcome and quality of life without increases in the percentage of deleted mtDNA are encouraging, we did not observe changes in mtDNA copy number. Therefore there remains a need for longer term studies to confirm that endurance exercise is a safe and effective treatment for patients with mitochondrial myopathies. The effects of detraining clearly implicate physical inactivity as an important mechanism in reducing exercise capacity and quality of life in patients with mitochondrial myopathy.

Keywords: mitochondrial myopathy; deletions; exercise training

Abbreviations: COX = cytochrome c oxidase; mtDNA = mitochondrial DNA; RPE = ratings of perceived exertion; SDH = succinate dehydrogenase

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Introduction

Defects of mitochondrial oxidation are important causes of neurological disease (Zeviani and Di Donato, 2004). The clinical features in these patients are highly variable with

both neurological and systemic involvement, although neurological features often predominate (McFarland *et al.*, 2002). One of the best characterized neurological features is

the muscle involvement, which may be severe in some patients and associated with significant fatigability and weakness.

The molecular defect in adult patients with mitochondrial myopathies commonly involves the mitochondrial genome, with either single, large-scale deletions or point mutations detected (Taylor and Turnbull, 2005). Since there are multiple copies of the mitochondrial genome in individual muscle fibres, these defects can be either homoplasmic (all copies of the genome mutated) or heteroplasmic (with a mixture of wild-type and mutated copies in the same muscle fibre). In the presence of heteroplasmy there is typically a threshold level of mutated mitochondrial DNA (mtDNA) required before a biochemical defect is observed. Since there is variation among different muscle fibres in the amount of mutated mtDNA, this leads to a mosaic picture of respiratory chain deficiency in muscle biopsies. This is best detected using histochemical techniques for cytochrome *c* oxidase (COX), (which has three catalytic subunits encoded by the mitochondrial genome) and succinate dehydrogenase (SDH) (all subunits encoded by the nuclear genome) (Sciaccio and Bonilla, 1996).

At present there are limited therapeutic interventions for patients with mitochondrial myopathies (Chinnery and Bindoff, 2003). Exercise training has been suggested as an approach to improve physical capacity and quality of life but whether it offers a safe and effective treatment for patients with heteroplasmic mtDNA mutations is currently uncertain. Endurance training was proposed as a therapeutic strategy to improve severe exercise intolerance in patients with high levels of mutation within skeletal muscle (Taivassalo *et al.*, 1996, 1998). The rationale was 2-fold: to counter adverse physiological effects of deconditioning caused by habitual avoidance of activities that provoke symptoms of fatigue; and to ameliorate the disease process by promoting an increase in mitochondrial biogenesis and muscle oxidative capacity. While adaptations to such training are well-established in healthy humans, effects of endurance training in the unique setting of mitochondrial heteroplasmy are difficult to predict. The goal of training in this case is to promote expansion of wild-type mtDNA, but equal or greater expansion of mutant mtDNA could also occur.

Studies of endurance training in patients with various heteroplasmic mtDNA mutations have demonstrated increased exercise and oxidative capacity associated with an improved quality of life (Taivassalo *et al.*, 1997, 1998, 2001; Siciliano *et al.*, 2000; Cejudo *et al.*, 2005). However, the only study to examine the effects of endurance training on the proportion of mutant versus wild-type mtDNA (Taivassalo *et al.*, 2001) found that, despite improved exercise and mitochondrial oxidative capacity, the percentage of mutant mtDNA increased in over half of the subjects, including patients with various heteroplasmic mtDNA mutations (single, large-scale deletions and point mutations affecting tRNA or protein-coding genes). These findings were interpreted to suggest that the overall volume of

wild-type mtDNA increased but that levels of mutant mtDNA increased disproportionately. These results have raised the concern that, despite short term physiologic and metabolic benefits, exercise training could contribute to the progression of mitochondrial disease by promoting an increase in mutation levels. Thus the question of whether training promotes a progressive expansion of mutant mtDNA needs to be resolved before the basic question of whether exercise should be encouraged or avoided can be answered (Taivassalo and Haller, 2004).

A critical corollary question relates to the consequences of habitual inactivity and to effects of cycles of greater and lesser regular physical activity levels in patients with mtDNA mutations. Exercise avoidance results in decreased physical capacity associated with reduced cardiovascular and skeletal muscle function in healthy humans and in a variety of disease states (Saltin and Gollnick, 1983). We have hypothesized that in patients with mitochondrial myopathies, deconditioning reduces mitochondrial volume, further restricting the capacity for oxidative phosphorylation and setting into motion a vicious cycle of worsening exercise tolerance. However, the physiological effects of deconditioning in patients with mitochondrial myopathies have never been directly assessed, and the critical question of how inactivity or cycles of greater and lesser physical activity levels affect the abundance and distribution of mutant versus wild-type mtDNA in skeletal muscle is currently unknown.

The objectives of this study were to assess the effects of exercise training and detraining in patients with sporadic single, large-scale mtDNA deletions. We chose to study these patients because single large-scale deletions are one of the most common forms of sporadic mtDNA mutations and usually result in an exclusively or predominantly skeletal muscle phenotype. We studied (i) the efficacy and safety of endurance training (14 weeks) in this patient population; (ii) the effect of more prolonged (total of 28 weeks) exercise training upon muscle and cardiovascular function and (iii) the effect of detraining (14 weeks) upon muscle and cardiovascular function. In each condition of training, prolonged training and deconditioning we evaluated physiological changes as well as effects upon the proportion, distribution and copy number of mutant and wild-type mtDNA in skeletal muscle.

Material and methods

Patient description

Eight patients (5 F: 37 ± 8 years; 3 M 46 ± 11 years) with molecular evidence of a sporadic single, large-scale deletion of mtDNA were enrolled in this study (Table 1). Exercise intolerance was experienced by all patients to a varying degree (mild to severe).

Study design and training protocol

The study was approved by the institutional review boards of University of Newcastle upon Tyne, University of Texas Southwestern Medical Center and Presbyterian Hospital of Dallas. Each patient was informed of the nature and risks of the study and gave

Table 1 Clinical and genetic characteristics of eight patients with single, large-scale mtDNA deletions

Patient	Age/sex	Clinical presentation	Size of mtDNA deletion (kb)
1	40/F	CPEO severe exercise intolerance, muscle weakness	4.2
2	40/F	CPEO, severe exercise intolerance	5.0
3	40/M	CPEO, mild exercise intolerance	4.8
4	36/F	CPEO, exercise intolerance	5.0
5	60/M	CPEO, exercise intolerance	3.6
6	45/F	CPEO, exercise intolerance, muscle weakness	4.2
7	25/F	CPEO, hearing impairment, retinitis pigmentosa, exercise intolerance	3.0
8	40/M	CPEO, exercise intolerance	6.0

**Fig. 1** Schematic of study design. After 14 weeks of training, eight patients were randomly selected to undergo continued training or discontinue training for an additional 14 weeks. Asterisk denotes evaluation of outcome measures.

their written consent to participate. As shown in the study design (Fig. 1), all patients underwent heart rate monitored exercise training on a stationary bicycle for a total of 49 sessions in 14 weeks (three times per week 1–7, four times per week 8–14). Exercise was performed at a training intensity corresponding to 70–80% of their maximal heart rate (determined at baseline as their maximal heart rate attained during the maximal exercise test) for 30 min per session for the first 7 weeks and 40 min per session for the last 7 weeks of training. Heart rates and exercise duration were continuously recorded on a Polar NV chest band and watch, allowing for subsequent analysis using a Polar/PC computer interface. Just prior to the 14 weeks evaluation, patients were randomized to either continue endurance training for an additional 14 weeks (at a training intensity and duration similar to weeks 8 through 14), or stop training and resume pre-study activity levels for 14 weeks. Exercise testing evaluation and muscle biopsy samples were obtained at baseline, 14 and 28 weeks.

Exercise testing

Each patient was assessed over a period of 5 days during which time two different methods of exercise testing were performed: maximal exercise testing to determine peak exercise capacity and physiological response (1 and 5 days); and submaximal exercise to determine exercise tolerance (day 3). All patients were familiarized with the experimental setup prior to testing on an electronically braked, pedal rate-independent cycle ergometer (MedGraphics, 2000). The maximal exercise test was performed twice for

repeatability, and data were averaged (when same peak workload was reached) or the higher workload of the two is presented. To ensure exercise testing (and training) did not induce muscle injury, creatine kinase (CK) levels were monitored prior to testing on day 1 and at the end of the week of testing on day 5.

Maximal exercise testing

As described previously, the workload was increased in 5–10 W increments every 1–2 min until the patient reached maximal heart rate (220 – age) or exhaustion, usually between 8 and 12 min. Gas exchange and cardiac output were determined at rest, during one or more submaximal workloads and with peak exercise. Expired air was collected in Douglas bags for 120 s at rest and 60 s during exercise for determination of $\dot{V}O_2$. The fractions of O_2 , CO_2 and N_2 in each bag were analysed with a Marquette 1100 Medical Gas Analyzer and ventilation was measured with a Tissot spirometer for calculation of $\dot{V}O_2$. Cardiac output (Q) was measured utilizing acetylene rebreathing in which the rate of disappearance of C_2H_2 from a rebreathing bag is proportional to pulmonary blood flow and Q. Determination of $\dot{V}O_2$ and Q allows for the calculation of systemic arterio-venous oxygen difference, indicated by the Fick equation: $\dot{V}O_2 = Q \times \text{systemic a-v}O_2 \text{ difference}$. $\Delta Q/\Delta \dot{V}O_2$, determined by linear regression, was used to assess the relationship between O_2 delivery and uptake. In healthy individuals, irrespective of age, sex, body weight or level of conditioning, there is a virtual 1:1 relationship between O_2 delivery and utilization as indicated by the fact that Q increases ~ 5 l for each litre of increase in O_2 consumption ($\Delta Q/\Delta \dot{V}O_2 \sim 5$) from rest to maximal exercise. When $\dot{V}O_2$ is limited by impaired muscle oxidative phosphorylation $\Delta Q/\Delta \dot{V}O_2$ is characteristically exaggerated (Taivassalo *et al.*, 2003). Heart rate was continuously monitored during rest and exercise with a 12-lead electrocardiogram (Quinton 3040 ECG monitor). All subjects had intravenous catheters inserted in a cubital vein from which blood was drawn for analysis at rest, various submaximal exercise levels and at the maximal workload. Whole blood samples were assayed for lactate using a commercially available analyser (Yellow Springs Instruments).

At the 14 and 28 week evaluations, the incremental workload protocol was identical to each patient's baseline evaluation. If the end-workload could be surpassed after a period of training, the exercise intensity was increased until same absolute pre-training heart rate was attained, and the corresponding maximal workload and physiological responses were noted.

Submaximal exercise testing

Exercise tolerance was assessed during constant workload exercise. Patients cycled at a workload corresponding to 60% of the maximal heart rate measured during the baseline maximal cycle test for 30 min or until exhaustion. Venous blood lactate was sampled at rest and at 10 min intervals during the exercise, heart rate was monitored throughout. Also during the test, the subjects' rating of exercise effort was obtained at the end of every minute using the two common Borg scales: the 15-point (ratings between 6 and 20) for overall body effort; and the 10-point (ratings between 1 and 10) for leg effort. These validated scales are widely used to gauge ratings of perceived exertion (RPE) and have been shown to correspond closely with metabolic and physiologic indices of increasing exercise intensity (Borg *et al.*, 1985). Testing was repeated using the same workload as the baseline evaluation at the 14 and 28 week evaluation.

Quality of life

Assessment of the effects of training and detraining on health, quality of life and physical functioning was obtained by a reliable and validated survey, the Short Form Health Survey Questionnaire (SF-36[®]) by the Medical Outcomes Study (Ware and Sherbourne, 1992). This standardized health status auto-assessment was completed by each patient. Scores of eight different components of the SF-36[®] were aggregated and tabulated to give a single physical component summary score, used to assess the self-determined improvement with exercise training per patient. The components reflect physical well-being (physical functioning, role-physical and bodily pain), psychological well-being (role-emotional and mental health) and combination of both (general health, vitality and social functioning). As a point of reference, the score for the general healthy US population, standardized for age and gender, is 50 ± 10 .

Muscle needle biopsy

At baseline, 14 weeks of training and after continued training or detraining, a needle biopsy of the mid-portion of vastus lateralis muscle was performed to obtain on average a 200 mg sample. These samples were immediately divided into sections, frozen and stored in liquid nitrogen for molecular genetic determinations, or frozen in isopentane cooled by liquid nitrogen for histological and histochemical analysis. Samples for each patient from each time point (baseline, 14 and 28 weeks) were analysed concurrently.

Histochemistry

Cryostat sections (10 μm) were cut from transversely orientated muscle blocks. Sections were reacted for both COX and SDH. The SDH section was used to determine the number of fibres with high levels of enzyme activity—equivalent to the so-called ‘ragged red’ fibres. Sequential assay of COX and SDH activities was used to identify COX-deficient fibres (Taylor *et al.*, 2004).

Biochemistry

The activities of the individual respiratory chain complexes I, II and IV were measured in a post 600g_{av} muscle supernatant and expressed relative to the activity of the matrix marker enzyme citrate synthase and to the wet weight of muscle homogenized (Taylor and Turnbull, 1997).

Mitochondrial DNA studies

The level of deleted mtDNA in each biopsy was determined by Southern blot analysis of total muscle DNA, the accepted ‘gold standard’ method for quantifying levels of mtDNA rearrangements. Briefly, total muscle DNA was digested with either PvuII or with SnaBI and probed with a PCR generated probe (15 782–1289 nt) that hybridize to the non-coding control region (Blakely *et al.*, 2004). Long-range PCR was used to amplify across the major arc using primers [L6249 (6249–6265 nt) and H16215 (16 225–16 196 nt), prior to using a panel of M13-tagged PCR primers to determine the precise deletion breakpoint in each patient. Sequencing of appropriate PCR products was performed using BigDye[®] Terminator v3.1 chemistries (Applied Biosystems) on an ABI 3100 automated DNA sequencer.

Real-time PCR

Fresh frozen muscle sections (20 μm) were mounted on PEN (polyethylenephthalate) slides (Leica Microsystems, Milton

Keynes, UK) and subjected to dual COX/SDH histochemistry as described above, and air-dried after dehydration. Groups of muscle fibres (COX-positive, COX-deficient and mixed populations) were cut into sterile 0.5 ml PCR tubes using a Leica Laser Microdissection (AS-LMD) System. Following centrifugation (7000 g_{av} for 10 min), the cells were lysed in 10 μl of cell lysis buffer [50 mM Tris-HCl (pH 8.5), 1 mM EDTA, 0.5% Tween-20, 200 ng/ml proteinase K] at 55°C for 2 h and then 95°C for 10 min to denature the proteinase K. For the studies on COX-positive and COX-deficient muscle fibres, 20 individual fibres were microdissected from the section. For the studies on mixed group of fibres an area (290 000 μm^2) was cut from each section. This represented between 12 and 25 fibres depending on the size of the fibres, and was cut at random through the section.

Quantitative real-time PCR approach and fluorogenic probes were used to calculate both the percentage level of deleted mtDNA and mtDNA copy number. PCR primers and fluorogenic probes (Applied Biosystems, Warrington, UK) for regions of *MTND1* (forward primer, L3485–3504; reverse primer, H3553–3532; probe, L3506–3529) and *MTND4* (forward primer, L12087–12109; reverse primer, H12170–12140; probe, L12111–12138) were synthesized, and 5 μl of DNA lysate was amplified separately with each of the *MTND1* and *MTND4* primer/probe combinations as described previously (He *et al.*, 2002; Bender *et al.*, 2006).

Statistical analysis

To evaluate the effects of training in all eight patients, a paired-*T* test was performed for comparison at baseline and 14 weeks. To distinguish the effects of continued training from detraining post-randomization of the patients into two groups (continued training and detraining), the difference between 14 and 28 weeks was calculated per individual in each group and the means of each group were compared using a two-sample *t*-test. This enabled the testing of whether the two groups differed in their response during the period between 14 and 28 weeks. By utilizing the delta for each measure, potential bias from the distribution of patients into either group was avoided. The small sample size per group ($n = 4$) after 14 weeks lowered the power needed to detect significant differences. Non-parametric statistics were applied to detect differences in quality of life scores before and after training

Results

Training compliance

All patients adhered to the training protocol over the duration of the study. The group of eight patients completed 47 ± 4 of the proposed 49 sessions over the 14 week training period at the intended exercise heart rate intensity, which corresponded to $75 \pm 5\%$ of their maximal heart rate (average maximal heart rate determined at baseline exercise testing was 172 ± 14 b.p.m.). The four patients who continued training completed 40 ± 4 sessions in the subsequent 14 week training period. Furthermore, no exercise-related injury or complications were experienced as shown by normal CK levels throughout the training (baseline average CK: 234 ± 236 U/l; 14 weeks training: 211 ± 132 U/l; 28 weeks training: 209 ± 135 U/l). Exercise testing also had no effect as revealed by serial CK determinations during the week of evaluation (baseline: maximal Test 1 = 234 ± 236 ,

Test 2 = 199 ± 211 ; 14 weeks training: Test 1 = 211 ± 132 , Test 2 = 176 ± 136 ; 28 weeks: Test 1 = 209 ± 135 , Test 2 = 201 ± 127 U/l).

Exercise physiology

Training effect

Fourteen weeks of endurance training significantly increased peak work capacity (26%), peak oxygen uptake (11%), and peak capacity for oxygen extraction (peak systemic a-v O_2 difference, 11%) (Table 2). There was no effect on peak systemic oxygen delivery (cardiac output, 2%). There was a trend for normalization of exaggerated cardiac output relative to oxygen utilization ($\Delta Q/\Delta VO_2$, 11% decrease $P = 0.056$). Patients also demonstrated an improved capacity for

Table 2 Physiological effects of 14 weeks of training in eight patients with mitochondrial myopathy due to single, large-scale mtDNA deletions

Outcome measure	Baseline	14 weeks
Maximal exercise		
Peak watts	85.6 ± 26	$107.5 \pm 32^*$
Peak VO_2 (l/min)	1.36 ± 0.4	$1.51 \pm 0.4^*$
Peak Q (l/min)	14.4 ± 3.9	14.7 ± 4.1
Peak A- VO_2 diff (ml O_2 /dl)	9.7 ± 2.5	$10.8 \pm 2.7^*$
$\Delta Q/\Delta VO_2$	8.5 ± 2.1	7.6 ± 2.8
Submaximal exercise at constant workload		
Blood lactate (mM)	3.5 ± 0.7	$2.2 \pm 0.6^{**}$
Heart rate (b.p.m.)	141 ± 17	$123 \pm 16^{**}$
RPE (overall body)	13 ± 1	$9 \pm 2^{**}$
RPE (leg)	4 ± 1	$2 \pm 1^*$
Quality of life		
SF-36 score	37 ± 7	$43 \pm 7^*$

* $P < 0.05$.

** $P < 0.01$.

submaximal exercise, as detected by a 1.3 mM mean decrease in blood lactate, an 18 b.p.m. decrease in heart rate (Fig. 2) and significantly lower RPE at the same constant workload after training (Table 2). Overall, the patients detected a significant improvement in quality of life after training with an increase in score (16%) from 37 ± 7 to 43 ± 7 after training (population norm score ~ 50). The specific components of the summary score that showed the greatest improvements were physical functioning (37 ± 7 to 43 ± 8 , contributing most heavily to physical well-being) as well as vitality (39 ± 13 to 45 ± 12) and general health (33 ± 10 to 39 ± 10), which contribute equally to both physical and psychological well-being.

Effects of continued training versus detraining

Effects upon peak work capacity, oxygen utilization and oxygen extraction differed significantly in the group that continued training compared to the group that underwent detraining (Table 3).

Continued training in four patients resulted in the general maintenance of peak work capacity (115 ± 41 W at 14 weeks to 126 ± 44 W at 28 weeks) and peak VO_2 (1.69 ± 0.45 to 1.72 ± 0.45 l/min) and an increase in peak a-v O_2 difference (10.3 ± 1.7 to 12.0 ± 2.9 ml/dl; $P < 0.05$) (Fig. 3). Likewise, indices of improved submaximal exercise capacity were maintained at 28 weeks in this group (Fig. 4); there was no further decrease in blood lactate (2.3 ± 0.8 mM at 14 weeks to 2.4 ± 0.7 mM at 28 weeks) or heart rate (118 ± 11 at 14 weeks to 120 ± 10 b.p.m. at 28 weeks). Patients' perception of overall (RPE = 9 ± 3 at 14 weeks; 9 ± 3 at 28 weeks) and leg effort (RPE = 2 ± 2 at 14 weeks; 2 ± 1 at 28 weeks) during submaximal exercise did not change. Quality of life scores remained the same during this period (43 ± 10 at 14 weeks to 43 ± 8 at 28 weeks).

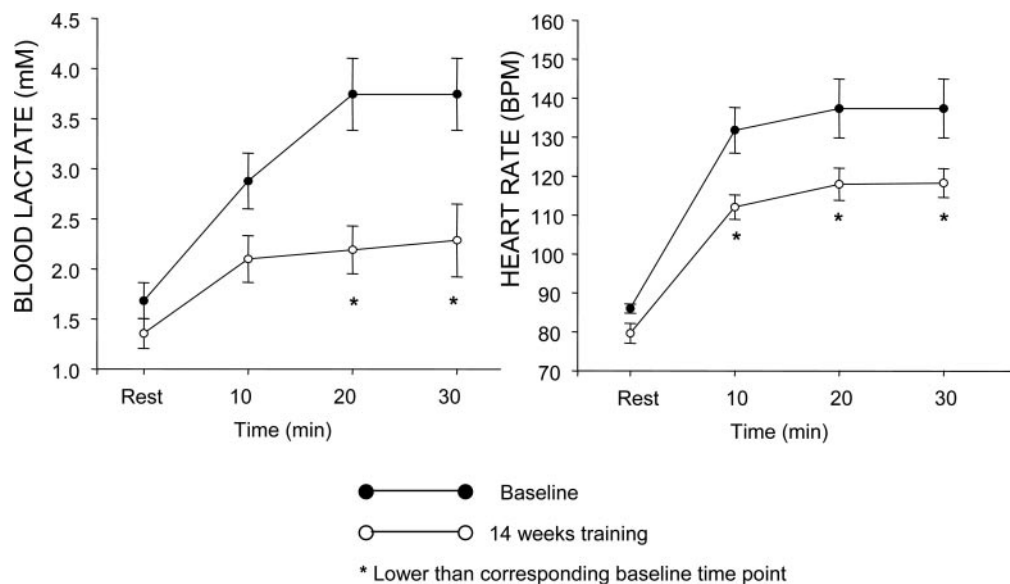
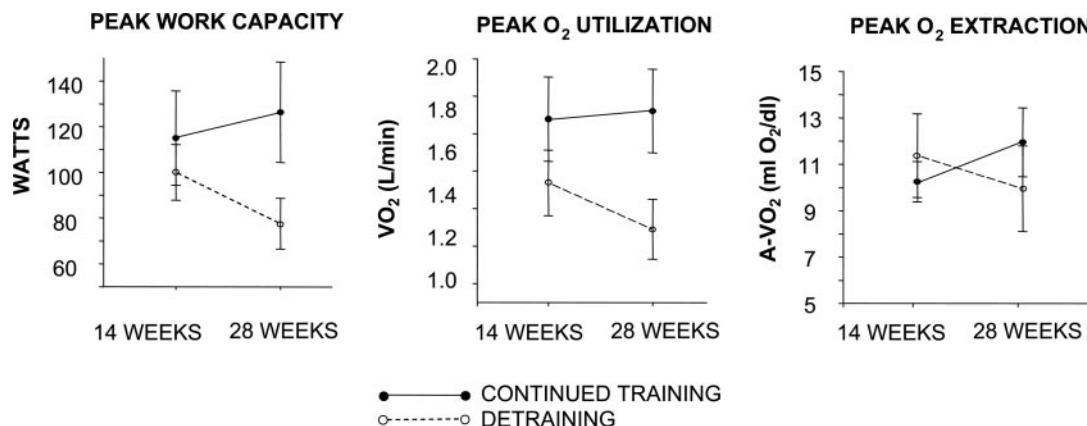
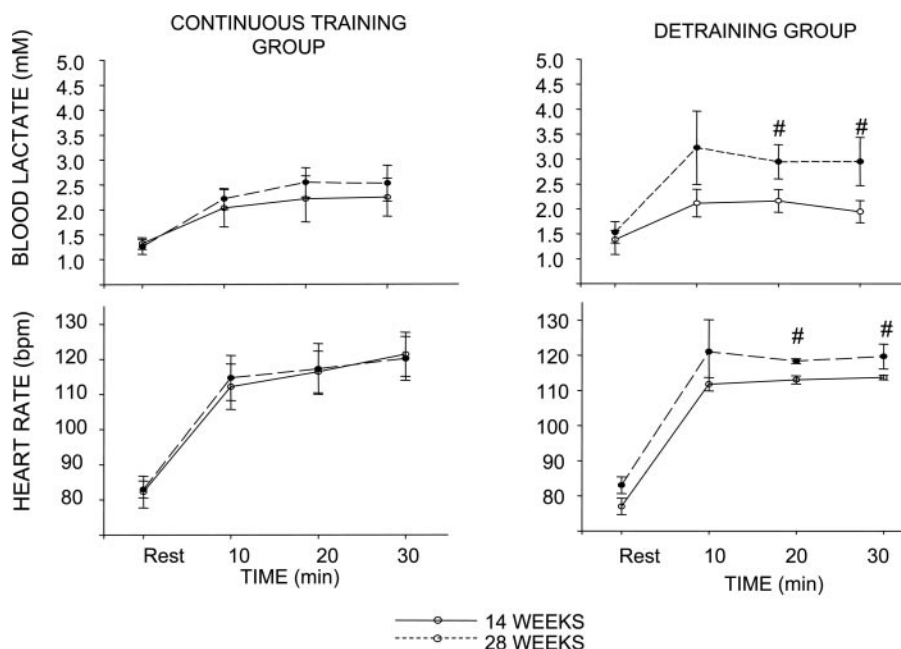


Fig. 2 Changes in blood lactate (left) and heart rate (right) during 30 min of constant workload submaximal exercise before and after 14 weeks of training in eight mitochondrial myopathy patients.

Table 3 Comparison of physiological changes after 14 weeks with continued training or discontinued training

Outcome measure	Comparison to trained state (Δ 14–28 weeks)		
	Continued training (n = 4)	Detraining (n = 4)	Difference between groups
Peak watts	$\Delta 11 \pm 10$ W	$\Delta -23 \pm 10$ W	$P = 0.003$
Peak VO_2	$\Delta 0.04 \pm 0.05$ l/min	$\Delta -0.25 \pm 0.12$ l/min	$P = 0.005$
Peak A- VO_2	$\Delta 1.7 \pm 1.7$ ml/dl	$\Delta -1.4 \pm 0.9$ ml/dl	$P = 0.014$

**Fig. 3** Physiological changes in maximal exercise capacity after 14 weeks of training, followed by further training or deconditioning, in patients with mitochondrial myopathy.**Fig. 4** Physiological changes in exercise tolerance during submaximal workloads after 14 weeks of training in the two subgroups of mitochondrial myopathy patients. Note that data shown from the detraining group are for three patients (denoted by #) as one patient was unable to complete >10 min of submaximal exercise at 28 weeks.

Patients who stopped training demonstrated the opposite response (Fig. 3); there was a mean decrease from the trained state in peak work (100 ± 24 W at 14 weeks to 78 ± 22 W at 28 weeks; $P < 0.05$), peak VO_2 (1.34 ± 0.32 to 1.09 ± 0.32 l/min; $P < 0.05$), and peak a- VO_2 diff (11.4 ± 3.6 to 10.0 ± 3.7 ml/dl;

$P < 0.05$). There was a trend for loss of adaptation during submaximal exercise as evidenced by a 1.1 ± 1.0 mM increase in blood lactate (2.1 ± 0.4 to 3.2 ± 1.3 mM) and $12 \text{ b.p.m.} \pm 14$ increase in heart rate (115 ± 2 to 127 ± 15) during constant workload after the cessation of training

(Fig. 4). This same absolute workload was perceived by the patients as requiring greater effort, indicated by increases in overall body RPE (9 ± 2 at 14 weeks; 12 ± 3 at 28 weeks) and leg effort RPE (2 ± 1 at 14 weeks; 4 ± 2 at 28 weeks). The group reported a significant decrease in quality of life after detraining compared to the trained state (43 ± 3 before, 35 ± 7 after detraining, $P < 0.05$). As a note, one patient in this group (Patient 1) injured her knee at the onset of the detraining phase (unrelated to the previous exercise training programme) and became significantly immobile, such that her physical activity was reduced to levels lower than prior to entry into the study. This patient was unable to complete >10 min of submaximal exercise at the 28 week evaluation (Fig. 4).

Interestingly, at the end of detraining, patients in this group had returned to their baseline with respect to peak exercise capacity: peak work capacity baseline = 76 ± 19 W, post-detraining = 78 ± 22 W; peak oxygen utilization baseline = 1.12 ± 0.28 , post-detraining 1.09 ± 0.32 l/min; peak oxygen extraction baseline = 9.7 ± 3.3 , post-detraining 9.9 ± 3.6 ml/dl. The mean submaximal exercise blood lactate at 28 weeks was equal to that at baseline assessment (baseline = 3.3 ± 0.6 , post-detraining 3.2 ± 1.3 mM) and mean submaximal heart rate was slightly lower (baseline = 135 ± 6 , post-detraining 127 ± 15 b.p.m.).

Histochemical analysis of biopsies

After 14 weeks of training there was no significant change in the percentage of COX-deficient fibres in the 8 patients overall ($19.1 \pm 12.7\%$ at baseline, 18.3 ± 14.3 at 14 weeks). Similarly there was no significant change observed in the percentage of hyper-reactive fibres ($5.1 \pm 6.1\%$ at baseline, $3.1 \pm 2.3\%$) after 14 weeks of training. In the group of patients that continued to train no change in the percentage of COX-deficient fibres was detected ($16.5 \pm 8.5\%$ at

14 weeks; $18.1 \pm 7.1\%$ at 28 weeks), whilst in the patients that stopped training the percentage of COX-deficient fibres increased from $20 \pm 19.8\%$ at 14 weeks to $27.4 \pm 34.9\%$ at 28 weeks, but this increase was entirely due to the results from Patient 1 (see Discussion).

Biochemical analysis of biopsies

After 14 weeks of training in the eight subjects there was a trend towards increases in the activity of respiratory chain complexes compared to the pre-training values (see Fig. 5A), compatible with previous studies suggesting increased mitochondrial oxidative activity. Whilst there was considerable variation, there was an apparent trend (not significant) for an increase in citrate synthase activities in the patients who continued to exercise compared to those who ceased training (Fig. 5B).

Molecular genetics

Level of mutated mtDNA

In all patients single, large-scale deletions were detected with no evidence of duplication. Minor variations in levels of mutant mtDNA were detected in individual patients after 14 weeks of training (five demonstrating a slight decrease, one a slight increase from pre-training levels, Table 4). The slight fall in overall level of deleted mtDNA observed after 14 weeks of training was not significant and within experimental error (mean level of deletion at baseline = $56 \pm 19\%$ post-training $53 \pm 20\%$). In the group that continued training the level of deleted mtDNA also did not change significantly from baseline ($56 \pm 18\%$) compared to the level after 28 weeks of training ($55 \pm 23\%$). Similarly, in the patient group that stopped training, there was no change in the level of deleted mtDNA between baseline ($55 \pm 23\%$), 14 weeks of training ($55 \pm 22\%$) and at 28 weeks with detraining ($55 \pm 26\%$).

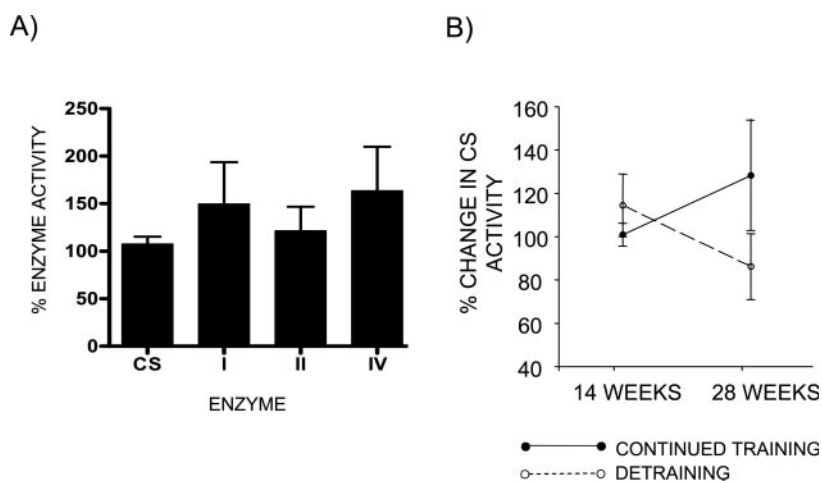


Fig. 5 Changes in respiratory chain enzyme complexes I, II and IV and citrate synthase activities with training. **(A)** The mean change in the group of eight mitochondrial myopathy patients (\pm SE) in enzyme activity of biopsy sampled after 14 weeks of training relative to baseline sample. **(B)** The mean change in citrate synthase activity in the two subgroups that underwent continued training or detraining.

Table 4 Percentage of mtDNA deletion as determined by Southern blot analysis in individual patients at baseline, following 14 weeks of training and after an additional 14 weeks of training or detraining

Subgroup (14–28 weeks)	Patient	Baseline	14 weeks	28 weeks
Continued training	2	75	73	76
	3	37	28	46
	4	67	63	64
	5	46	38	33
Discontinued training	1	87	87	87
	6	39	39	32
	7	55	52	64
	8	39	43	37

Real-time PCR studies of deletion levels and copy number

To try to gain further insight into the mechanisms involved in the effects of exercise on the mitochondrial genome in the presence of a heteroplasmic mtDNA defect, we also evaluated the mutation levels and copy number of mtDNA in collections of COX-deficient, COX-normal or mixed fibres using a real-time PCR technique. This assay has been previously validated on muscle and has also proved valuable in quantifying mtDNA deletions in other tissues (He *et al.*, 2002; Bender *et al.*, 2006). As one patient in our cohort (Patient 5) harboured an mtDNA rearrangement in which the *MTND4* gene was not deleted, this patient was excluded from all real-time analysis. To assess overall copy number in the biopsy, three samples of mixed fibres were analysed in triplicate and the mean value determined. The copy number per unit area did not significantly change after 14 weeks of training when compared to baseline values (2.2 ± 1.0 copies per μm^2 baseline, 2.0 ± 0.6 copies after 14 weeks training). No significant change in copy number was seen after continued training or detraining (data not shown).

The level of deleted mtDNA in COX-deficient muscle fibres was significantly different compared to the COX-normal fibres as would be expected (COX-deficient $98 \pm 2\%$; COX-normal $55 \pm 20\%$ $P > 0.001$). No change was observed in the percentage level of deleted mtDNA following exercise in mixed (baseline $74 \pm 15\%$, after training at 14 weeks $73 \pm 15\%$), COX-deficient fibres (baseline $98 \pm 1\%$, after 14 weeks training $98 \pm 1\%$) or COX-normal fibres (baseline $57 \pm 13\%$, after 14 weeks training $51 \pm 11\%$).

Discussion

This study evaluated the effects of training and detraining in a group of mitochondrial myopathy patients harbouring a common, sporadic mutation in mtDNA, a single large-scale deletion. The major findings were that: (i) 14 weeks of exercise training improved submaximal exercise tolerance and peak capacity for work, oxygen utilization and skeletal muscle oxygen extraction with no change in the level of deleted mtDNA; (ii) continued training for an additional

14 weeks maintained these beneficial adaptations; (iii) the cessation of training (detraining) resulted in loss of physiological adaptation to baseline capacity with no overall change in mutation load as determined by Southern blot and real-time PCR.

Physiological responses

Effects of 14 weeks of training

The ability of endurance training to improve exercise capacity and quality of life in patients with mitochondrial myopathy was confirmed. Training increased peak work (26%) and oxygen utilization (11%). The dominant physiological mechanism of increased oxygen utilization was an increase in peak systemic arterio-venous O_2 difference (11%) consistent with an enhanced capacity of muscle mitochondrial oxidative phosphorylation. As we have described previously (Taivassalo *et al.*, 2003), the major physiological manifestation of impaired muscle mitochondrial function during exercise in this patient population is a blunted ability to increase the extraction of oxygen from blood, as indicated by an abnormally low peak systemic a-v O_2 difference. Conversely, the circulatory response during exercise is 'hyperdynamic', where delivery of oxygen (cardiac output) is exaggerated relative to oxygen utilization ($\Delta\text{Q}/\Delta\text{VO}_2 > 5$). This exercise response contrasts to that of healthy individuals, in whom systemic a-v O_2 difference increases 3-fold from rest (~ 5 ml/dl) to peak exercise (~ 15 ml/dl), and cardiac output is tightly matched to oxygen utilization, so that $\Delta\text{Q}/\Delta\text{VO}_2 \sim 5$. Peak baseline exercise and oxidative capacity of the mitochondrial myopathy patients was less than half of age-predicted values for sedentary healthy individuals, but the percentage increase with training was within the range expected for healthy individuals undergoing similar training. However, the mechanism underlying this improvement differs from the training response in healthy individuals. Normally, increased capacity for oxygen delivery (cardiac output), is the major physiological basis of improved exercise capacity with training whereas increases in peak systemic O_2 extraction (a-v O_2 difference) are relatively minor. In mitochondrial myopathy patients, we have demonstrated that the mechanism underlying improved exercise capacity relates predominantly to improved ability for oxygen extraction by skeletal muscle. In this study, peak systemic a-v O_2 difference increased on average from 9.7 ± 2.5 to 10.8 ± 2.7 ml/dl, a result that closely parallels our previous findings. In contrast to training effects in healthy subjects, there were no changes in peak capacity for oxygen delivery over the 14 weeks of training, with cardiac output remaining remarkably constant (baseline 14.4 ± 3.9 ; post-training 14.7 ± 4.1 l/min).

In addition to improving peak exercise capacity, endurance training increased patients' tolerance of submaximal exercise. During the same workload after training, blood lactate levels were 1.3 mM lower, consistent with enhanced oxidative phosphorylation. The corresponding

heart rate was on average 18 b.p.m. lower, and patients' perception of effort was reduced. Together, these adaptations are consistent with a training-induced increase in capacity for skeletal muscle oxidative phosphorylation and would be expected to improve patients' ability to perform activities of daily living. This notion is supported by patient self assessment of significantly improved quality of life with respect to physical well-being, general health and vitality after training. Findings from the submaximal exercise testing clearly demonstrate that moderate physical activity is far better tolerated after training.

The current study extends previous physiological and molecular findings of endurance training effects in a group of 10 patients with heterogeneous mtDNA defects, by focusing on a more homogeneous group of mitochondrial myopathy patients with a uniform mtDNA molecular defect. The previous study included only two patients with single large-scale deletions and the others with various tRNA and protein-coding mutations (Taivassalo *et al.*, 2001). Patients in the current study had a lower mean mutation load (~55%) compared to the previous study (~65%), with only 3 patients harbouring >65% levels compared with two-third of the previous subjects having mutation levels >65%. Accordingly, in the current study, baseline exercise and oxidative capacity were higher, as was peak systemic a-vO₂ difference during cycle exercise, a surrogate marker of muscle capacity for oxidative phosphorylation (Taivassalo *et al.*, 2003), (9.7 ± 2.5 ml/dl compared to the previous patient group 6.9 ± 2.6). Nevertheless, the magnitude of improvements in work and oxidative capacity achieved after endurance training was similar in both studies. Although, the mean increase in maximal oxygen uptake was 20% in the previous study (versus 11% in the current study), the increases in maximal oxidative capacity for the two patients with single large-scale deletions were 10 and 16%, similar to our current findings.

Effects of continued training

This phase of the study represents the longest period of training reported thus far in patients with mitochondrial myopathy. Although the number of patients studied in this phase was small, findings indicate that the physiological benefit of training was maintained through 28 weeks. The training volume was similar to that of the first 14 weeks of training, and whether further improvement in exercise capacity and quality of life might be achieved with more intense exercise or even a longer period of training awaits further study.

Effects of detraining

For the first time, the physiological effects of detraining in mitochondrial myopathies have been quantitatively assessed. Fourteen weeks of detraining following an equal duration of training resulted in a loss of beneficial physiological adaptations. Interestingly, the magnitude of increase from

baseline was equal to the magnitude of decrease from the trained state. For example, peak work capacity increased by 24 ± 8 W with training, and fell by 23 ± 10 W with detraining, peak oxygen utilization increased 0.22 ± 0.17 l/min and decreased 0.25 ± 0.12 l/min, and peak oxygen extraction increased 1.7 ± 1.0 ml/dl with training and decreased 1.5 ± 0.9 ml/dl with detraining. At the end of detraining, patients in this group were back to their baseline physiological condition. Detraining effects were magnified in one patient who most dramatically reduced physical activity levels to below baseline (Patient 1).

Muscle histochemistry, biochemistry and molecular genetics

Effects of training

The physiological improvements following 14 weeks of training were associated with a trend toward increases in citrate synthase and respiratory chain complex activities which, however, were not as marked as in our previous study and did not achieve statistical significance. Nevertheless, we consider this trend toward increase oxidative enzyme activities along with the consistent training related increase in systemic a-vO₂ difference to be compatible with an increase in muscle capacity for oxidative phosphorylation. The strength of this study over previous studies is the homogeneous nature of the genetic defect, but in spite of this there was considerable difference in the severity of the biochemical, histochemical and clinical abnormalities.

Improved muscle oxidative capacity was not associated with increases in the overall percentage of mutant mtDNA, unlike our previous study in which variable increases and no decreases in mutant mtDNA were detected in a heterogeneous group of molecular mtDNA defects, including two patients with single, large-scale deletions, raising concern about the safety of endurance training as a potential therapeutic approach. The minor decreases and increases in mutant mtDNA detected in the present study are consistent with expected results from sampling a heterogeneously distributed mtDNA mutation. In muscle containing lower overall mutation loads, variability in mtDNA and COX-deficient fibre content between serial needle biopsies is expected to be greater than in patients with higher mutation loads and more uniformly distributed COX-deficient fibres. Despite the lower mutation load in the current study and issue of variability, no significant increases in the level of deleted mtDNA after training were detected. In addition, no consistent changes in the percentage of COX-deficient fibres were associated with training. A previous study has highlighted how difficult it is to accurately quantify the COX deficiency in both large and small muscle biopsies (Barron *et al.*, 2005). Variation occurs both between and within muscle groups, even when counting up to 400 muscle fibres. Our data and those of previous studies therefore have to be interpreted cautiously.

We have shown using two different methods (Southern blotting and real-time PCR) that no increase in the level of deleted mtDNA following endurance training was detected. The relative lack of change in mtDNA copy number was surprising and may relate to the smaller changes in biochemical measures and mitochondrial biogenesis. Whilst mtDNA copy number correlates with measures of citrate synthase activity in general (Wang *et al.*, 1999), there are few studies describing the anticipated effect of endurance training on the magnitude and relationship between mtDNA replication, biogenesis and enzyme activity in healthy humans.

Effects of detraining

There were no consistent changes in the level of deletion or distribution of COX-deficient fibres with discontinued training in the group on average. Interestingly however, a dramatic change was observed in Patient 1 who underwent detraining. This patient had 48% COX-deficient fibres at baseline and after 14 weeks of training, but this increased markedly when she stopped training to 79% COX-deficient fibres. A knee injury acquired immediately post-training, unrelated to exercise, caused this patient to become significantly immobile and to limit physical activity to levels below that of her baseline condition. Despite this extreme form of deconditioning no change was observed in the percentage of deleted mtDNA in all three biopsies (87% deleted mtDNA in all three). Of note, however, was the finding of a 40% decrease in the copy number of total mtDNA in the COX-deficient fibres in the final biopsy. This would support evidence that the COX deficiency relates to the amount of wild-type molecules rather than the total percentage of mutated mtDNA.

In conclusion, findings from this study provide further substantial evidence that endurance exercise training has many potential benefits for patients with mitochondrial myopathies. The training volume (intensity, frequency and duration) applied in this study specifically to patients with single large-scale mtDNA deletions resulted in significant physiological changes that led to improved maximal and submaximal exercise capacity and enhanced quality of life. Furthermore, over the course of a 28 week training period, we did not detect any evidence of increased mutation load. Whilst these results are encouraging, we remain cautious about extending these results to other mtDNA mutations and advising exercise training as treatment. The lack of more significant changes in both the biochemical measurements and copy number of mtDNA with this type of training protocol warrants further investigation into training-induced mitochondrial proliferation and effects on mutation level in single fibres. There is a need for long term studies to assess whether the physiological benefits can be maintained over a longer period of time, and more importantly, that no change in mtDNA mutation load occurs. Our findings on the effects of detraining clearly implicate physical inactivity

as an important mechanism in reducing exercise capacity and quality of life in patients with mitochondrial myopathy and underlie the urgency of clarifying endurance training effects at the molecular level.

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