Published in final edited form as:

Med Sci Sports Exerc. 2014 October ; 46(10): 2030–2036. doi:10.1249/MSS.0000000000000316.

Method for Controlled Mitochondrial Perturbation during Phosphorus MRS in Children

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Abstract

Introduction—Insulin resistance (IR) is increasingly prevalent in children, and may be related to muscle mitochondrial dysfunction, necessitating development of mitochondrial assessment techniques. Recent studies used ³¹Phosphorus magnetic resonance spectroscopy (³¹P-MRS), a non-invasive technique appealing for clinical research. ³¹P-MRS requires exercise at a precise percentage of maximum volitional contraction (MVC). MVC measurement in children, particularly with disease, is problematic due to variability in perception of effort and motivation. We therefore developed a method to predict MVC, using maximal calf muscle cross-sectional area (MCSA) to assure controlled and reproducible muscle metabolic perturbations.

Methods—Data were collected from 66 sedentary 12–20 year-olds. Plantar flexion-volitional MVC was assessed using a MRI-compatible exercise treadle device. MCSA of the calf muscles were measured from MRI images. Data from the first 26 participants were utilized to model the relationship between MVC and MCSA (predicted MVC = 24.763+0.0047*MCSA). This model was then applied to the subsequent 40 participants.

Results—Volitional vs. model-predicted mean MVC was 43.9±0.8 kg vs. 44.2±1.81 (P=0.90). ³¹P-MRS results when predicted and volitional MVC were similar showed expected changes during volitional MVC-based exercise. In contrast, volitional MVC was markedly lower than predicted in 4 participants, and produced minimal metabolic perturbation. Upon repeat

testing, these individuals could perform their predicted MVC with coaching, which produced expected metabolic perturbations.

Conclusions—Compared to using MVC testing alone, utilizing MRI to predict muscle strength allows for a more accurate and standardized ^{31}P -MRS protocol during exercise in children. This method overcomes a major obstacle in assessing mitochondrial function in youth. These studies have importance as we seek to determine the role of mitochondrial function in youth with IR and diabetes and response to interventions.

Keywords

^{31}P -MRS; muscle mitochondria; children; exercise perception

INTRODUCTION

The prevalence of insulin resistance (IR) and type 2 diabetes mellitus (T2D) are increasing in children, potentially related to obesity driven alterations in mitochondrial function (27). IR is associated with decreased mitochondrial function in T2D adults, as well as in aging, burn trauma and HIV medication-related lipodystrophy (10, 11, 29). Exercise improved mitochondrial function as well as insulin sensitivity in adults with T2D, yet weight loss did not change rates of mitochondrial oxidative phosphorylation in obese men (16, 22, 37). As such mitochondrial function in disease states needs to be measured to determine whether it is affected by interventions. Few studies have assessed the role of mitochondrial function in children with IR or T2D, in part due to methodological limitations. Such studies are critical in the effort to develop future preventive and therapeutic strategies targeting mitochondria in this at-risk group.

Traditionally, muscle biopsies have been used for assessment of mitochondrial function. Methods include measuring concentrations and activities of enzymes necessary in TCA cycling or oxidative phosphorylation, such as citrate synthase or succinate dehydrogenase, assessment of mitochondrial number by mitochondrial DNA or electron microscopy or quantification of respiration rates in isolated muscle fibers. Samples analyzed with these methods demonstrate increased markers of oxidative capacity in athletes, and decreased markers in individuals with obesity, T2D or severe trauma (10, 11, 35). However, muscle biopsies are invasive and generate small tissue samples which may not be representative of the entire muscle area (15). Further, mitochondrial function may be affected by substrate delivery and relative mitochondrial location in the cell, which may be disrupted during biopsy (9, 26). Thus, mitochondrial biopsy study results are useful to answer certain mechanistic questions, but cannot be fully predictive of *in vivo* mitochondrial function.

Measuring mitochondrial phosphorylation rates via ^{31}P phosphorus magnetic resonance spectroscopy (^{31}P -MRS) is a non-invasive technique that requires no ionizing radiation exposure and allows *in vivo* assessment of mitochondrial oxidation following a physiologic perturbation. Furthermore, muscle ATP, phosphocreatine and free phosphate concentrations are directly measureable at rest, during a perturbation such as exercise and during recovery from exercise via ^{31}P -MRS. ADP concentrations can also be calculated during recovery from exercise and can be used to model *in vivo* oxidative phosphorylation (2). ^{31}P -MRS

methodologies require a controlled metabolic perturbation, and exercise protocols including both isotonic and isometric exercises have been utilized in quadriceps, calf and forearm muscles (3, 19, 21).

In-magnet exercise protocols require an accurate pre-assessment of muscle strength to determine an appropriate workload that is comparable between individuals. Previous studies found that isometric exercise rates at 70% or less of maximal volitional contraction (MVC) are optimal. These workloads cause a metabolic perturbation without excessive fatigue or dropping the intramuscular pH below 6.9, a level known to alter ATP kinetics (2, 21, 25, 40). However, determining a true MVC can be problematic due to several factors including variable motivation, sense of fatigue and degree of fitness, and these confounders are amplified in youth due to the incomplete development of their neurologic systems (6, 7). If an accurate MVC is not determined, results can be skewed, as ADP recovery time is faster following a lower work load. These errors are amplified when comparing populations with different exercise tolerance thresholds, such as trained compared to untrained populations.

The goal of this study was to develop a method to validate volitional MVC in youth in order to ensure equivalent workloads are prescribed to subjects during in-MRI exercise, as well as to assess mitochondrial activity via ^{31}P MRS.

METHODS

66 pediatric participants aged 12–20 years were recruited from pediatric clinics at the Children's Hospital Colorado and the Barbara Davis Center for Childhood Diabetes for a prospective, cross-sectional study. Participants included normal weight controls, obese controls, participants with type 1 diabetes (T1D), T2D or polycystic ovarian syndrome (PCOS). All participants were untrained, with less than 3 hours per week of exercise (as verified by standardized 3-day activity recall, and 7-day accelerometer, Acti-graph, Pensacola, FL), and had achieved Tanner Stage 2 or above in puberty. All Tanner staging was performed by K.J. Nadeau or M. Cree-Green, Pediatric Endocrinologists. PCOS was diagnosed per NIH criteria with adolescent adaptation: oligomenorrhea defined as <8 menses a year, at least 2 years after menarche and clinical or biochemical signs of hyperandrogenism.

Participants underwent a screening visit, and an exercise/imaging study visit which included MRI imaging of the leg for cross-sectional area (MCSA) and in-MRI exercise testing with ^{31}P -MRS. All MRS testing was performed fasting, with no strenuous exercise for the 3 days prior, verified by questioning the subject and parents. Participants were also instructed to refrain from caffeine the day of the study. This study was approved by the University of Colorado Anschutz Medical Campus Institutional Review Board and the Children's Hospital of Colorado Scientific Advisory Review Committee. Informed consent was obtained from all participants 18–20 years old, and parental consent and participant assent was obtained from all participants less than 18 years old.

Plantar Flexor Maximal Volitional Contraction Strength Testing and Exercise

Strength testing was done on a custom- built MR-compatible plantar flexion treadle device with force measurement capability similar to exercise apparatuses previously described (4, 19). In brief, the exercise bench consists of a foot pedal 15 cm long, mounted at 15 degrees plantar flexion. When pressure is applied, the foot pedal interacts with a force transducer box. Force is measured 10.6 cm above the heel, a distance approximately half that of the foot length, and designed to minimize the contribution of foot muscles to the measured force output. The force transducer box is connected to an external read-out stage (Omega Engineering, Stamford, CT), and finally to a laptop computer for constant recording of force throughout exercise with DAQ software (Labview, National Instruments, Austin TX).

Participants were placed supine on the exercise board with the dominant foot strapped to a foot pedal with 3 separate 4.5 cm straps. The thigh was secured to the exercise bench immediately above the knee and a second strap was applied at the hip. Participants were asked to cross their arms across their chest during the exercise to minimize any upper body contribution through pushing against the magnet housing or exercise bench. Participants were verbally coached on how to achieve maximal force for exercise and how to isolate the muscle of interest. Participants then practiced while being observed by study personnel. Once participants demonstrated the correct technique, the MVC was measured as a mean of 3–5 contractions for 5–10 seconds each, with 30 seconds of rest between attempts.

The ^{31}P -MRS exercise protocol consisted of measurements during rest for 90 seconds, isometric plantar flexion exercise for 90 at 70% MVC, and recovery for 10 min post-exercise. Force was monitored continuously throughout the exercise, and participants verbally coached to help keep the force measurements within the target goal. The average force applied was recorded in kg. All participants were able to complete the exercise for 90 seconds at or near target force.

Magnetic Resonance Imaging and Spectroscopy

Imaging Acquisition—Imaging and spectroscopy were performed on a General Electric (GE) 3 Tesla magnet (GE, Milwaukee, WI) upgraded with GE spectroscopy research software. A custom $^1\text{H}/^{31}\text{P}$ leg coil was used for imaging and spectroscopy (Clinical MR Solutions, Brookfield, WI). The coil is a concentric probe made of an inner coil 9 cm in diameter (for ^{31}P) and a 13 cm outer coil tuned to ^1H frequency for scout imaging and shimming. Initial scout images were obtained for measurement of maximal gastrocnemius and soleus cross sectional area (MCSA), with protocol settings of an echo time (TE)=2.8 ms, repetition time (TR)=225 ms, field of view (FOV)=16 mm and slice thickness of 4 mm, and a slice separation of 1 mm for 20 slices. The area imaged included from the proximal end of the tibial tuberosity to well below the maximal area of the gastrocnemius.

Imaging Analysis—Gastrocnemius and soleus MCSA was quantified by manually tracing the external edges of these muscles electronically using GE advantage workstation software (GE, Milwaukee, WI). This technique was repeated in at least 5 of the 20 slices, to identify the slice with MCSA. A representative picture from one of our participants is shown in Figure 1.

Spectroscopy—Rates of mitochondrial phosphorylation were assessed by ^{31}P MRS performed at 51.70 MHz with the $^1\text{H}/^{31}\text{P}$ coil. The machine was auto shimmed with ^1H , then a ^{31}P scan was performed for resting baseline measurements (long TR of 15,000, flip angle of 135 degrees and 32 scans) to measure a fully relaxed spectrum. The ^{31}P exercise scan was then performed under partially saturated conditions (TR 1000, TE 3000, flip angle 135, 2048 pts).

Spectroscopy Analysis—Peak positions and areas of interest [phosphocreatine (PCr), inorganic free phosphate (Pi), β -ATP(3 peaks), α -ATP(2 peaks), γ -ATP(2 peaks), and PME] were determined by time domain fitting using jMRUi (18, 39) utilizing AMARES (A Method of Accurate, Robust and Efficient Spectral fitting), a nonlinear least-square-fitting algorithm using previously built prior knowledge files (32). The ratio of soleus Pi:PCr was measured as previously described (45, 46). All exercise scan spectra were corrected for saturation using the fully relaxed spectra for that day.

Statistical Analysis

Data are expressed as mean $\pm$ standard deviation unless otherwise stated. Wilcoxon rank sum tests and ANOVA with Tukey-Kramer post-hoc p-value adjustments were used to test differences in continuous variables. Fisher's exact test was used to test the difference in distribution of sex and Tanner Stage among groups. Multiple linear regression was used to determine the association between volitional MVC with MCSA and predicted MVC. Inter-rater reliability coefficient (ICC) was used to test the agreement between volitional and predicted MVC and a Bland-Altman plot to assess bias. The effect of BMI category and sex on the relationship between MVC and MCSA were assessed within the entire cohort with a two-sided Student's t-test. Analyses were performed using SAS 9.3 (Cary, NC) and GraphPad Prism 5.1. $p < 0.05$ was considered statistically significant.

Model Development

Data from spectra from 26 youth were used to create a predictive equation based on MCSA and volitional MVC. Subject characteristics included: 12 normal weight and 14 obese; 8 had PCOS, 6 T1D, 5 T2D and 7 were normal controls; 19 females and 7 males. Subject mean age was 15.1 ± 2.2 years. Mean MCSA was $4180.0 \pm 1089.8 \text{ cm}^2$ and volitional MVC was $44.3 \pm 7.9 \text{ kg}$. The linear regression equation was $\text{MVC} = 24.763 + 0.0047 * \text{MCSA}$ (Figure 2A).

Participants for model application

In the validation sample of 40 participants, 12 were normal weight and 28 were obese; 10 were normal controls, 15 had PCOS, 6 had T1D, and 9 had T2D. MCSA, volitional MVC and predicted MVC were higher in obese participants than in normal weight participants (Table 1). In univariate analysis, BMI accounted for 60% of the variability in MCSA and only 8% of the variability in volitional MVC. MCSA and predicted MVC were not different by disease status after adjusting for BMI.

RESULTS

The initial model equation listed above was applied to the data from the 40 validation participants to calculate predicted MVC. Predicted MVC was significantly correlated with volitional MVC, ($\beta = 0.005 \pm 0.001$, $p < 0.0032$, $R^2 = 0.2073$, Figure 2B). In a model adjusted for sex, Tanner Stage, and BMI, predicted MVC was more closely related to volitional MVC ($\beta = 0.0054 \pm 0.0023$, $p = 0.0228$, $R^2 = 0.43$), and only predicted MVC was significant ($F=5.72$, $p=0.022$), with no significant contribution from the other variables. Predicted MVC tended to over-estimate volitional MVC ($\beta = 0.996 \pm 0.316$, $p = 0.0032$, $R^2 = 0.2032$) and the Intra class correlation was 0.344; however, there was no significant bias. When the entire group of participants was examined, the relationship between MCSA and MVC did not differ by obesity status (Normal weight versus Obese, $p = 0.8860$; Figure 2C) or sex (female versus male, $p = 0.7569$; figure 2D).

Four participants had a volitional MVC that was much lower than their predicted MVC, and had minimal metabolic perturbation during a subsequent in-MRI exercise (isometric contraction at 70% of their volitional MVC for 90 seconds), indicating that their measured volitional MVC was not really a MVC. In-MRI exercise testing was repeated on a separate day in these participants, instead using 70% of their predicted MVC as the goal, which all participants were able to achieve with coaching, again demonstrating that their original MVC testing underestimated their true ability. The predicted MVC-based exercise produced the expected metabolic perturbation.

DISCUSSION

Reproducible non-invasive measures of mitochondrial function are needed for the evolution of mitochondrial function in youth. To assess mitochondrial function *in vivo* with ^{31}P MRS, we needed to create a standardized exercise induced metabolic perturbation in youth. We developed an equation to accurately predict an adolescent's plantar flexion MVC, based on their calf MCSA as measured by MRI. Application of this equation to a validation cohort of adolescents showed that the equation was robust regardless of BMI, Tanner stage, sex. Application of the predicted MVC when children performed sub-maximally on volitional MVC tests allowed for correction of workload and a systematic exercise perturbation between individuals. Although a similar method has been reported in adults for the calf muscle, and in the forearm in youth, these results cannot be generalized to the calf in youth, nor to youth with obesity or diabetes (1, 13, 22, 28). Thus our data provide the first available predictive equation for assessing MVC in the calf muscle based on MRI measured maximum CSA in youth with and without diabetes and obesity.

We had anticipated that obese youth may have a different relationship between MCSA and volitional MVC compared to normal weight youth, however there was no difference between the two groups. In obese participants, MCSA includes extensive visually evident extramyocellular lipid, as well as intramyocellular lipid in those obese participants who are significantly insulin resistant (12, 24). Thus, MCSA in obese participants includes not only active muscle tissue, but also variable amounts of lipid which may cause the predicted MVC to over-estimate strength. These potential confounders cannot be distinguished and corrected

for with the rapid MRI method needed to predict the MVC in real-time. However, despite these potential confounders, there was not a systematic overestimation of MVC in our obese participants we studied.

We also did not find a significant impact of either sex or Tanner stage on the relationship between MCSA and MVC. This is anticipated, as sex steroids, specifically testosterone, cause muscle hypertrophy in conjunction with improved strength. Thus in those subjects with a later Tanner stage or in males, the MCSA will be larger as will the MVC. Improvements in muscle strength efficiency, i.e. increased strength for a similar muscle mass, are typically thought to develop as a training effect, not in relation to testosterone exposure *per se*. With our current model, the sex steroid effects on muscle are thus accounted for in the measurement of MCSA.

The relationship between volitional MVC and muscle area for the cohort was similar in our study to those calculated in other muscle groups in youth and adults. When assessing forearm strength in children, forearm MVC was found to correlate best with forearm maximal muscle volume (38). In young, active boys, the knee extensor and flexor MCSA correlated well with both isometric and isotonic contractions, and this relationship remained stable for 6 months (30). Studies in untrained adult women found that both the calf muscle physiologic MCSA and the anatomical MCSA correlated with MVC ($R^2=0.511$ and $R^2=0.537$ respectively)(5). Akagi *et al* found a similar relationship between calf plantar flexors and force ($R^2=0.44$) in both male and female untrained participants, although the males tended to be stronger and have more muscle than the females (1). Our data thus appears to have a similar variability to what is published in other patient populations and other muscle groups, despite careful coaching and observation by trained personnel during exercise.

There has been limited research into children's perception of volitional exercise effort. Volitional effort is a complex process, with psychological and attentional components as well as physiological signaling involvement. With the brief, single leg exercise paradigm we are utilizing, physical factors such as respiratory or cardiac abnormalities are less likely to play a role, compared to non-specific mediators such as pain responsiveness or lactic acidosis (34). It is more likely that psychological factors such as expected level of performance, previous experience with exercise and personality traits influence our model of MVC exercise testing (23, 34). Further, ^{31}P -MRS has been used to investigate mitochondrial function in diabetes, and there is evidence that volitional exertion can vary with T1D or obesity in adolescents, and in adults with T2D (8, 17, 20, 28, 33). Thus, specifically when comparing these populations to controls, additional measures are needed to ensure that equal biochemical effort occurs during the exercise bout.

There are several ways to cause a metabolic perturbation, and then standardize across patient populations. The most standard method, used in most exercise model based studies, is the one that we have described, in which a subject performs multiple exercises to determine their volitional MVC. An alternative method, specific to MRS studies with a live spectra readout (Siemens Systems), calls for exercise to continue until the PCr peak decreases to 30 to 50% of the pre-exercise height (3). This type of control creates an exercise that perturbs

the participant's muscles to a similar metabolic level; regardless of how much work was needed to achieve this metabolic perturbation. However, this method is not available at institutions with other types of MRI machines. A third method involves external electronic stimulation of the muscle, via direct nerve stimulation (36). However, this method still requires calibration with the participant's volitional MVC, as a set voltage can cause very different responses in different patient populations (36). Further, this method can be painful, and can vary with electrode placement and thus is reserved for adult studies in which there is a question involving the function of the neuromuscular unit. A fourth method, typically used in patients with ischemia-related diseases such as peripheral artery disease, or externally induced ischemia by methods such as arterial occlusion, requires exercise to exhaustion (2, 13). This method works well in patients who fatigue early (typically 1–3 min of exercise), but in healthy controls it requires prolonged magnet time, which is costly (2). Further, if exercising to fatigue, there is an increased risk of the muscle pH dropping below 6.9, the threshold known to alter mitochondrial ATP generation. Finally, the perception of true fatigue is also fraught with the same exercise perception problems as discussed above, and may introduce even greater variability to the results, especially in children. Therefore, we feel that our method is optimal for youth, and for exercise protocols in which the investigators desire to produce equivalent work load based muscle perturbation; as opposed to simply working the muscle to a set metabolic perturbation level.

There are 2 published series of ^{31}P -MRS exercise in children, both using volitional MVC-related techniques, however, neither study is in the calf. In one series, a 30% MVC isometric leg extension was performed for a 3 minute period (14). Post-study analysis was done, and if the post-exercise pH was <6.95 , it was assumed that the exercise perturbation was adequate to assess ADP recovery. The benefit of using isometric exercise is that if one particular contraction fails to be of adequate force, the repetitive nature of the exercise allows for the cumulative effect of the efforts to be accounted for. However, movement can induce motion artifacts, and reduce the spectra signal to noise ratio. Another group performed ^{31}P MRS on the forearm, in boys and men, with a 15% MVC handgrip exercise, to stimulate the forearm flexors (31). The authors found a very tight correlation between the MVC and the flexor M-CSA ($R^2=0.93$). However, the mean post-exercise pH for both groups was 6.5, indicating that the work load was likely too intense to accurately assess ATP production rates. These findings highlight the importance of avoiding over-exercising the muscle, while still causing an adequate perturbation to allow for ADP generation. Our exercise paradigm allows for both of these, and the predictive equation aids in maintaining this balance.

A limitation of the broader applicability of our method is that we studied untrained, sedentary adolescents, since this reflects youth with obesity and T2D. Adults or trained individuals with greater muscle strength may require generation of a separate curve. However, a strength of our design is the inclusion of a variety of normal and disease-states (normal weight, T1D, T2D, obese, PCOS), making our curve potentially applicable to many adolescent patient populations.

In conclusion, by utilizing a predicted MVC to verify a subject's volitional muscle strength, researchers can ensure accurate results during a controlled exercise protocol in children;

even if a subject performs poorly on the MVC test itself or suffers from motivational issues. In our current protocol, we are now measuring the MVC and also calculating the predicted MVC. When there is >5% difference between the two, we now use the higher of the two to ensure that adequate exercise perturbation is achieved. These studies have relevance as we seek to determine mitochondrial function in youth with IR and diabetes as well as other metabolic diseases.

Acknowledgments

FUNDING/ACKNOWLEDGMENTS

K.J.N.: NCRR K23 RR020038-01, NIH/NCRR Colorado CTSI Co-Pilot Grant TL1 RR025778, NIH/NIDDK 1R56DK088971-01, JDRF5—2008-291, ADA 7-11-CD-08

M.C.G.: Thrasher Pediatric Research Foundation Mentored Pilot Grant, NIH/NCRR Colorado CTSI Co-Pilot Grant TL1 RR025778, Pediatric Endocrinology Fellowship training grant NIDDK T32 DK063687, Center for Women's Health Research Faculty Development Award.

JEER: VA Merit, Denver Research Institute, 5P01HL014985 and the Center for Women's Health Research.

JGR: American Diabetes Association Clinical Research Award; NIH Building Interdisciplinary Research Careers in Women's Health (PI) K12 HD057022; Center for Women's Health Research.

This research was also supported by University of Colorado Adult CTSC NIH Grant M01-RR00051, Pediatric CTSC NIH Grant 5M01-RR00069 and NIH/NCRR Colorado CTSI Grant UL1 RR025780.

The authors would like to the participants and their families for participating.

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Figure 1.
Tracing of Calf Maximal Cross Sectional Area

Figure 2.
Correlation with Volitional MVC

Table 1

Test Subject Demographics by BMI

	Lean (N = 12)	Obese (N = 28)	P-value
Age	15.17 ± 2.17	15.29 ± 1.72	9284
Gender (Female)	7 (58.3%)	27 (96.4%)	0.0060
Max Area	3136.75 ± 699.97	4495.32 ± 1012.33	0.0003
Volitional MVC	38.82 ± 10.17	46.51 ± 11.38	0.0513
Predicted MVC	39.51 ± 3.29	45.89 ± 4.76	0.0003
Tanner Stage 2	2 (18.2%)	0 (0%)	0.0169
Tanner Stage 3	1 (9.1%)	1 (3.6%)	
Tanner Stage 4	1 (9.1%)	0 (0%)	
Tanner Stage 5	7 (63.6%)	27 (96.4%)	