Validation of Musculoskeletal Ultrasound to Assess and Quantify Muscle Glycogen Content. A Novel Approach

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Abstract: Glycogen storage is essential for exercise performance. The ability to assess muscle glycogen levels should be an important advantage for performance. However, skeletal muscle glycogen assessment has only been available and validated through muscle biopsy. We have developed a new methodology using high-frequency ultrasound to assess skeletal muscle glycogen content in a rapid, portable, and noninvasive way using MuscleSound (MuscleSound, LCC, Denver, CO) technology. **Purpose:** To validate the utilization of high-frequency musculoskeletal ultrasound for muscle glycogen assessment and correlate it with histochemical glycogen quantification through muscle biopsy. **Methods:** Twenty-two male competitive cyclists (categories: Pro, 1–4; average height, 183.7 ± 4.9 cm; average weight, 76.8 ± 7.8 kg) performed a steady-state test on a cyclergometer for 90 minutes at a moderate to high exercise intensity, eliciting a carbohydrate oxidation of 2–3 $g \cdot min^{-1}$ and a blood lactate concentration of 2 to 3 mM. Pre- and post-exercise glycogen content from rectus femoris muscle was measured using histochemical analysis through muscle biopsy and through high-frequency ultrasound scans using MuscleSound technology. **Results:** Correlations between muscle biopsy glycogen histochemical quantification ($mmol \cdot kg^{-1}$) and high-frequency ultrasound methodology through MuscleSound technology were $r = 0.93$ ($P < 0.0001$) pre-exercise and $r = 0.94$ ($P < 0.0001$) post-exercise. The correlation between muscle biopsy glycogen quantification and high-frequency ultrasound methodology for the change in glycogen from pre- and post-exercise was $r = 0.81$ ($P < 0.0001$). **Conclusion:** These results demonstrate that skeletal muscle glycogen can be measured quickly and noninvasively through high-frequency ultrasound using MuscleSound technology.

Keywords: glycogen; biopsy; ultrasound; MuscleSound

Introduction

Carbohydrate (CHO) metabolism is important during exercise, particularly high exercise intensity, which is the predominant energy substrate for skeletal muscle. Glycogen is the storage form of glucose and CHO in mammals and humans. Carbohydrates are a limited source of energy, accounting for 1% to 2% of total bodily energy stores.[1] Furthermore, about 80% of total CHO is stored in skeletal muscle; 14% is stored in the liver; and 6% is stored in the blood in the form of glucose. This represents 300 to 400 g of glycogen that is stored in muscle and 70 to 100 g stored in the liver.[2,3] Studies have shown that glycogen depletion is associated with fatigue, decreased performance,[4–10] and increased risk for overtraining.[2,3,11] Exercise duration also plays an important role in CHO metabolism during

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exercise. Since glycogen storage capacity is about 400 to 500 g in the muscle and liver, exercise duration is critical for the regulation of CHO metabolism. Glucose uptake in skeletal muscle is dependent on glycogen content,[12] and hypoglycemia during exercise can be prevented by sufficient CHO intake.[13] Exercise duration is closely related to glycogen storage, as low amounts of glycogen during endurance events are associated with hypoglycemia, fatigue, and decreased performance,[4–10] even when other sources of energy are available.[14] Furthermore, glycogen is involved in the control of muscle contractility and force. Decreased glycogen stores are involved in decreases in skeletal muscle glycogen force, Ca^{2+} release, and myofibrillar protein function.[15–21]

Skeletal muscle glycogen assessment can aid in athletic performance via nutrition adjustment and athletic training. However, there are no practical and applicable methods of glycogen assessment for athletes. Muscle biopsy has been used to assess muscle glycogen content in the research field. The first percutaneous muscle biopsy can be accredited to French neurologist Guillaume-Benjamin Duchene, who developed the first percutaneous or semi-open biopsy needle to study muscle dystrophy and other muscle diseases.[22] Different muscle biopsy needles have been described in the literature.[23] However, the most commonly used percutaneous needle biopsy technique to measure muscle glycogen was introduced by Bergström in 1972[24] and has been modified over time.[25,26] This technique and the larger diameter trocar may endanger neurovascular structures. Therefore, this technique is limited to muscles with low vascularity (eg, vastus lateralis). An alternative to the Bergström technique has been the Weil-Blakesley Conchotome.[27–29] This instrument is considered safer because it does not require a sharp trocar to penetrate muscle. It opens the possibility of sampling different muscles with increased vascularity, such as the rectus femoris (RF). However, the techniques described are invasive and can have serious risks for complications. These techniques are not applicable for regular glycogen assessment, which would be ideal in the athletic population for the purpose of monitoring training and nutrition. A relatively new technique for glycogen assessment is nuclear 13C magnetic resonance spectrometry (13C MRS).[30–33] This methodology has the advantage of being noninvasive and allows assessment of glycogen content. However, like muscle biopsy, it is not an applicable method to athletes for regular glycogen assessment due to the long sampling protocols, expensive equipment, and lack of portability.

Materials and Methods

Subjects: Twenty-two male competitive cyclists with a valid US cycling racing license, professional and amateurs, categories 1 to 4 (Table 1[34]).

Study Design and Research Methods

The study was conducted at the Human Performance Laboratory of the Anschutz Health and Wellness Center at the University of Colorado School of Medicine in Aurora, CO. All study procedures were conducted in accordance with the Declaration of Helsinki, with a predefined protocol approved by all researchers and the Colorado Multiple Institutional Review Board, and with oversight from the University of Colorado Conflict of Interest Committee. All participants provided informed consent before any study procedures were performed.

Exercise Regime

In order to minimize fatigue and maximize glycogen storage levels, subjects were instructed not to exercise in the 48-hour period prior to the laboratory test. Subjects were also instructed not to perform $>$ 2 hours of moderate-intensity cycling in the 7 days prior to the laboratory test (Table 2).

Diet Monitoring

A dietitian prescribed a high-carbohydrate diet consisting of 8 g of carbohydrates per kg of the individual's body weight each day for the 3 days prior to the laboratory exercise test. These instructions were intended to optimize glycogen storage levels for day 6. In addition, subjects underwent muscle, biopsy, and ultrasound evaluation. Our dietitian instructed the subjects on the amount and types of food to ingest in the 3-day period before testing.

Blood Analysis

Complete blood count, creatinine kinase, and lactate dehydrogenase were measured 48 hours before the laboratory exercise test to exclude muscle damage that occurred prior to the test. It has been described that muscle damage can interfere and decrease muscle glycogen storage.[35,36]

Table 1. Subject Characteristics

Age, Years	Height, cm	Weight, kg	% Body Fat[34]	BMI, kg/m²	# Races/Year
31.3 ± 5.1	183.7 ± 4.9	76.8 ±7.8	12.1 ± 2.4	22.7 ± 1.8	27.1 ± 19.7

Abbreviation: BMI, body mass index.

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Table 2. Study Design

Day	Exercise Taper	High Carbohydrate Diet	Blood Test	No Exercise	Exercise Test, Muscle Biopsy Ultrasound
1	X				
2	X				
3	X	X			
4		X	X	X	
5		X		X	
6					X

Exercise Testing Protocol

All tests were performed at the Human Performance Laboratory at the Anschutz Health and Wellness Center. Subjects performed a 90-minute cycling test on an electromagnetically braked cyclergometer (Lode Excalibur Sport; Lode, Groningen, The Netherlands). Minute ventilation, oxygen consumption, and gas exchange were measured via ParvoMedics TrueOne 2400 Metabolic Measurement System (ParvoMedics, Inc., Sandy, UT). Heart rate was monitored via a heart monitor (Polar S725X; Polar Electro, Kempele, Finland). Exercise testing was performed at an exercise intensity eliciting between 2 and 3 g/min of CHO oxidation. Carbohydrate oxidation rates were calculated according to stoichiometric equations by Jeukendrup and Wallis.[37] Every 10 minutes during the test, a sample of blood was collected to analyze intra- and extra-cellular levels of L-lactate (YSI 1500 Sport; YSI, Yellow Springs, OH).

Assessing Muscle Glycogen via High-Frequency Musculoskeletal Ultrasound

Measurements were taken on each subject from the anterior superior iliac spine on the pelvis to the superior patellar pole. The midpoint on the thigh was marked, which generally corresponded to the midpoint of the RF and VL muscles. Using a 12 MHz linear transducer and a standard diagnostic GE LOGIQ-e ultrasound machine, scans of the right and left RF and vastus lateralis (VL) muscles were performed in sagittal (long-axis) and transverse (short-axis) planes. To reduce the possibility for compression artifact caused by inconsistent pressure with the transducer, the athlete contracted his quadriceps. In pilot studies, this has been shown to reduce examiner variability. At baseline, the skin was marked with a marker to ensure that postexercise scans were obtained in precisely the same location. These images were then wirelessly transmitted to a secure cloud-based Web application (MuscleSound, LLC, Denver, CO). This application can quickly process high-resolution DICOM images of specific muscles to create a quantifiable score of muscle glycogen content. Before the score was calculated, the image was pre-processed to isolate the muscle fibers under analysis. This was done by cropping the sides of the image where irregularities and artifact were common. The image was then blurred to remove noise and was changed to a binary (black/white) image. This method automatically identifies the skin, fat, and connective tissue within the muscle. The skin and tissues below the RF or VL were subtracted. The image was then returned to gray scale. The white connective tissues have a pixel intensity of 255. The remaining muscle tissue has a pixel intensity between 0 and 254. Once the muscle fibers in the image were isolated, the mean pixel intensity of the muscle was calculated, creating the glycogen score.

In the 22 subjects assessed, we found consistency in the ultrasound appearance of the right and left RF and the VL. We chose to focus more on the RF muscle because there is less connective tissue in the RF, which reduces potential artifact in the images. There is also less connective tissue in the muscle biopsy samples. By reducing these potential artifacts, we hoped to improve sensitivity and specificity, allowing us to detect more precise changes in muscle glycogen content. Another subjective reason for choosing the RF is the common belief by many competitive cyclists that this region of the quadriceps feels empty and fatigued before the lateral region of the thigh.

Ultrasound-Guided Muscle Biopsy Protocol

After the ultrasound scans were obtained, the right and left RF muscles were visualized and mapped in short and long axis to ensure that the biopsy was obtained from precisely the same location as the ultrasound glycogen score. Color flow Doppler was utilized to identify and avoid vascular structures. Biopsies were obtained using a Bard Monopty Disposable Core Biopsy Instrument 12 gauge × 10-cm biopsy needle (Bard Biopsy Systems, Tempe, AZ). Two passes of this device were done pre- and post-exercise under direct ultrasound guidance to ensure sampling of correct tissue (avoiding vessels) and sample quality (each pass obtained about 50 mg of muscle tissue).

At baseline, biopsies of the right RF were obtained under local anesthesia (2.5 mL of 0.25% bupivacaine; 5 mL of 1% lidocaine). The tissue was immediately frozen in liquid nitrogen and stored at –80°C for later analysis. Steri-strips closed the wound, and a pressure dressing was placed on the biopsy site using Elastoplast tape, allowing the cyclist to immediately exercise. After the test, the left RF (which had already been anesthetized) was biopsied in the same manner as we performed at baseline. The same type of pressure dressing was placed, and wound care instructions were given. By first sampling the right RF, we reduced risks for bleeding and hematoma formation, a complication that would have created artifact in the postexercise biopsy sample. Our complication rate was zero. No hemorrhages or hematomas were reported.

Glycogen Analysis of the Muscle Biopsy

The muscle glycogen concentration was determined according to the method described by Chan and Exton.[38] The 2 tissue samples of 50 mg each were immediately frozen in liquid nitrogen and stored at –80°C for later analysis. The freeze dried sample was powdered, dissected free of all visible non-muscle tissue, weighed, and subsequently digested by incubation in a 10-fold volume of 0.03 N hydrogen chloride for 10 minutes in a 100°C water bath. We used 50 mg of tissue in 500 µl of 0.03 N hydrogen chloride. Three 75-µl aliquots of the muscle homogenate were placed on individual 1.75 cm × 1.75 cm squares of Whatman 3M chromatography paper. The glycogen was then precipitated and immobilized onto the filter papers by washing three times in 33% ethanol dried in a laboratory oven. The fixed glycogen was then hydrolyzed into glycosyl units (glucose) and glucose by incubating the filter papers in 1 mL of amyloglucosidase solution at 37°C in a shaking water bath for 90 minutes at 90 revolutions per minute. The amount of glucose present was then quantitated using a glucose oxidase-peroxidase coupled enzymatic reaction and ELISA system. Briefly, 200 µl of a PGO/o-dianisidine solution was placed in wells of a 96-well microplate followed by 20 µl of hydrolyzed glycogen sample or glycogen standard. The plate was incubated at 37°C for 30 minutes. The reaction was stopped with the addition of 10 µl of 4 N hydrogen chloride. The microplate was then read on a spectrophotometric microplate reader at 420 nm. The concentration of glucose was measured versus a standard curve of glycogen standards ranging from 0 to 300 mg % glycogen/glucose. Final values were converted to mmol glycogen per kg muscle tissue.

Data Analysis Plan

The primary outcome was to compare the gold standard of muscle biopsy to the new approach using high-frequency ultrasound to determine muscle glycogen stores. We hypothesize that there would be no substantial difference in quantification of muscle glycogen stores at both pre- and post-exercise between biopsy and ultrasound. Comparisons between both groups in the study were done via a Student t test for independent data. The determination of the Pearson correlation coefficient was used to verify the existence of relationships between the different variables studied. Statistical significance was set at $P < 0.001$.

Results

Correlations between RF biopsy glycogen histochemical quantification (mmol·kg^{-1}) and high-frequency ultrasound methodology through MuscleSound technology were $r = 0.93$ ($P < 0.0001$) and $r = 0.94$ ($P < 0.0001$) for pre- and post-exercise, respectively (Figures 1 and 2). Glycogen content through histochemical analysis decreased from pre-exercise levels of 97.2 ± 34.1 mmol·kg^{-1} to 62.4 ± 22.8 mmol·kg^{-1} post-exercise ($P < 0.001$). Glycogen content through high-frequency ultrasound methodology through MuscleSound technology decreased from a score of 59.8 ± 15.9 pre-exercise (range, 0–100) to 39.8 ± 13.9 post-exercise ($P < 0.0001$). The correlation between muscle biopsy glycogen quantification and high-frequency ultrasound methodology for the change in glycogen from pre- and post-exercise was $r = 0.81$ ($P < 0.0001$; Figure 3). Since VL muscle has typically been used to perform blind muscle biopsy, we also established the correlations between RF muscle and VL muscle through MuscleSound technology, which were $r = 0.93$ ($P < 0.0001$) and $r = 0.91$ ($P < 0.0001$) for pre- and post-exercise, respectively (Figures 4 and 5). Finally, the correlation between RF and VL muscles for the change in glycogen from pre- and post-exercise was $r = 0.76$ ($P < 0.0001$; Figure 6).

Discussion

The published literature demonstrates that skeletal muscle glycogen and CHO availability are important for exercise performance. However, there has not been an efficient, rapid, and noninvasive method to assess and quantify muscle glycogen storage.[39] The methodology we have developed using high-frequency ultrasound with MuscleSound technology shows high correlations between the gold standard muscle biopsy with our ultrasound methodology for pre- and post-exercise.

Typically, the VL muscle has been used to perform muscle biopsy.[24,40] However, VL muscle is rich in connective tissue, which may interfere with histochemical and echographic quantification. Due to the lower amount of connective tissue in the RF muscle compared to the VL, we chose this muscle to evaluate via ultrasound and muscle biopsy. For this purpose, we have developed an ad hoc new method to perform ultrasound-guided muscle biopsy in the RF muscle utilizing the precise sampling of

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Figure 1. Correlation between MuscleSound score and muscle biopsy histological quantification of glycogen pre-exercise.

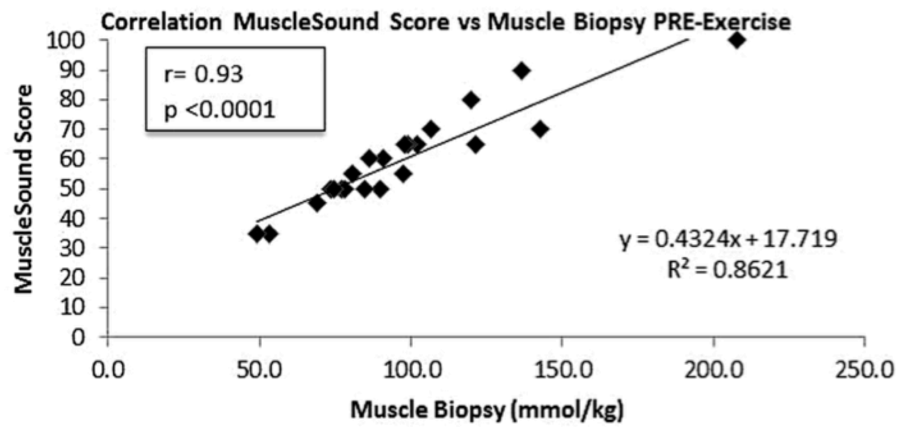


Figure 2. Correlation between MuscleSound score and muscle biopsy histological quantification of glycogen post-exercise.

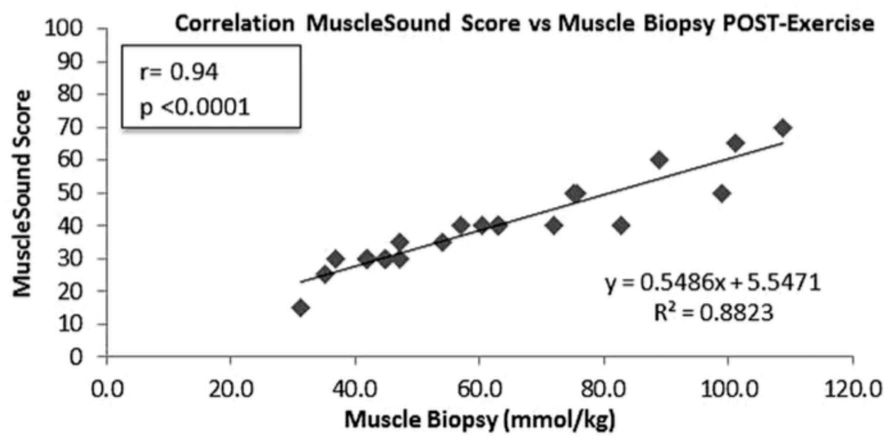
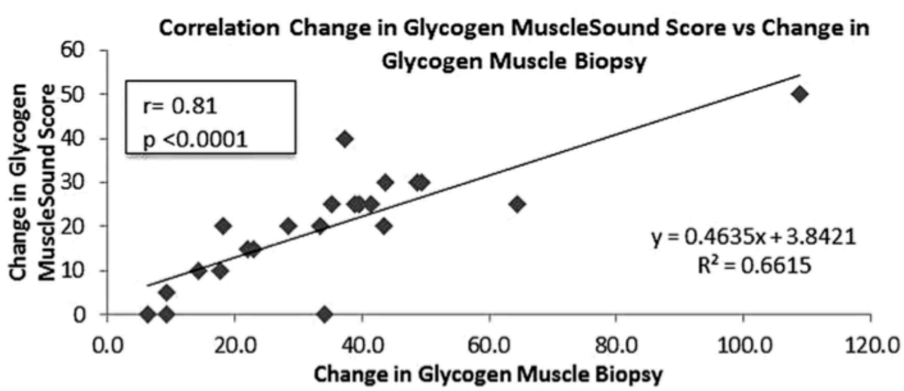


Figure 3. Correlation between muscle biopsy glycogen quantification and MuscleSound technology for the change in glycogen from pre- and post-exercise.



the Bard Monopty Disposable Core Biopsy Instrument 12 gauge × 10-cm biopsy needle. In our study, both the RF and VL muscles served as accurate sites for glycogen assessment through high-frequency ultrasound, as the correlation between RF and VL through MuscleSound technology was $r = 0.93$ ($P < 0.0001$) and $r = 0.91$ ($P < 0.0001$) for pre- and post-exercise, respectively.

Our methodology is valid to detect changes in the decrease of glycogen overtime. A decrease in skeletal muscle glycogen content during exercise has also been observed

Figure 4. Correlations between rectus femoris muscle and vastus lateralis muscle through MuscleSound technology pre-exercise.

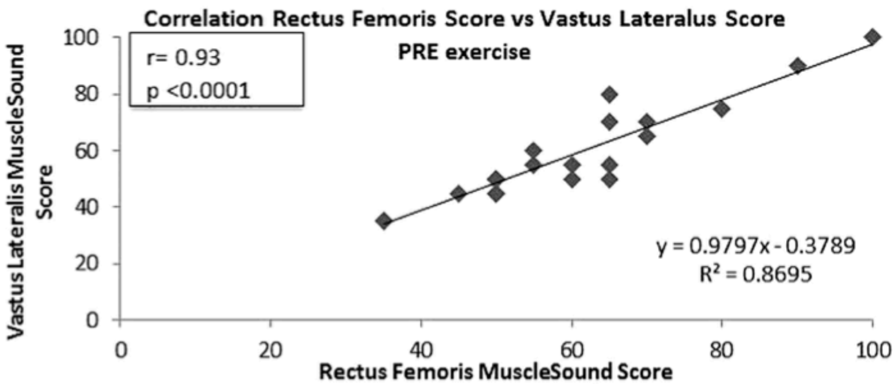


Figure 5. Correlations between rectus femoris muscle and vastus lateralis muscle through MuscleSound technology post-exercise.

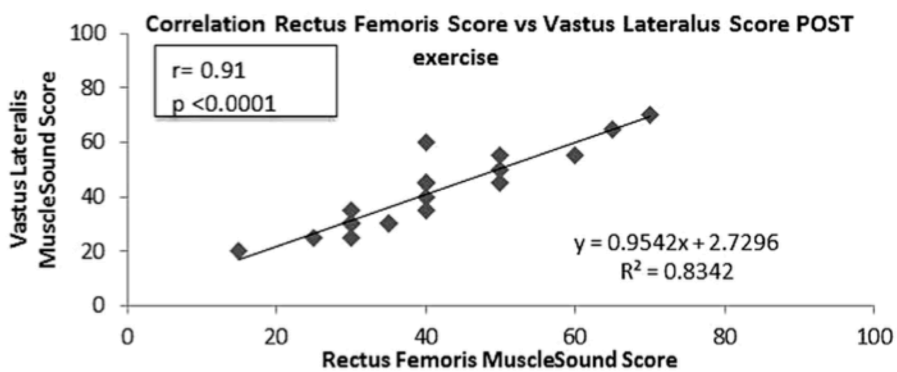
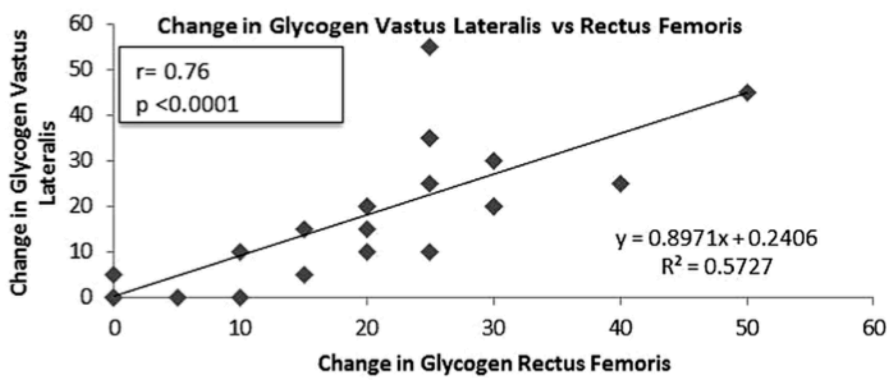


Figure 6. Correlation between rectus femoris and vastus lateralis muscles for the change in glycogen from pre- and post-exercise through MuscleSound technology.



in different studies.[4–10,41,42] In studies observing glycogen changes in cycling bouts,[4–8,10,42] changes in glycogen content vary, including ours, as duration and intensity of exercise are determinants for substrate utilization and glycogenolysis. It is important to note that the level of physical fitness is also involved in the regulation of substrate utilization. Well-trained subjects possess a higher fat oxidation capacity and rely less on glycogen. To our knowledge, our study is the first to use elite and competitive cyclists for the determination of skeletal muscle content pre- and post-exercise. We expected to observe lower glycogen depletion over time compared with previous studies that involved moderately active individuals or recreational athletes who rely more on CHO metabolism during exercise. Furthermore, we believe that quantifying the decreases in glycogen content through MuscleSound technology could be an important tool for athletic trainers, nutritionists, and coaches. Depending on how much glycogen an athlete has lost during exercise, it could be possible to estimate how tasking a given training has been for the athlete. In addition, it can help determine the best nutritional approach for replenishing skeletal muscle stores.

The correlation observed for the change in glycogen pre- and post-exercise between RF biopsy histochemical quantification and MuscleSound technology was 0.81 ($P < 0.0001$). We did not observe a decrease in pre- and post-exercise glycogen content via MuscleSound technology in 3 subjects. In 1 subject, we observed a small decrease in glycogen (0–5 score points). However, 3 of these 4 subjects also showed the lowest decreases in glycogen content through muscle biopsy histochemical quantification (6.2–9.3 mmol/kg). We observed via histochemical quantification that 1 subject showed a much higher decrease in glycogen compared with the other subjects (108.9 mmol/kg). Nonetheless, this same subject also had the highest decrease in glycogen through MuscleSound technology (50 points). Although some subjects could be interpreted as outliers (Figure 3), we believe these differences are possible due to the higher scale of the histochemical quantification of glycogen in mmol/kg (49.3–207.8 mmol/L) observed in this study) compared with the 0 to 100 scale of glycogen quantification through MuscleSound technology. It is also possible that the subjects who did not show a decrease or a small decrease in muscle glycogen content throughout the 90-minute test were efficient at oxidizing fat during exercise, preserving skeletal muscle glycogen content. This might be the case because similar muscle glycogen was measured via MuscleSound technology and histochemical quantification of muscle biopsy.

We found a good correlation for the change in glycogen through MuscleSound technology between RF and VL muscles for pre- and post-exercise ($r = 0.76$; $P < 0.0001$). A great advantage of our methodology is its portability. As part of our pilot data, we have collected extensive data in real competition situations with professional cycling, basketball, football, and baseball teams. Another benefit is the speed in measuring skeletal muscle glycogen. Within 15 seconds, it is possible to scan the muscle and quantify the amount of glycogen in skeletal muscle. With such rapid and accurate information, it is possible to make decisions on nutrition and training. Another great advantage of our methodology is its affordability compared with 13C MRS, which is the only other alternative for noninvasive glycogen assessment. This MRI methodology is difficult to find in most clinical settings and the cost can be prohibitive to athletes. The cost of MuscleSound technology based on ultrasound should be close to the cost of regular ambulatory skeletal muscle ultrasound scans, making it more affordable than 13C MRS.

The applications of our novel methodology may impact critical care and other fields of medicine. Often, injury and infection elicit a marked increase in glucose utilization, which can cause glycogen depletion.[43] Our methodology could open new doors for monitoring the muscle glycogen content and therefore nutrition in critically ill patients. Space medicine physiology is another field that might benefit from our methodology. Due to the technical and logistical limitations, performing skeletal muscle biopsies during space flights or microgravity is not practical. To our knowledge, there are no research studies on substrate utilization and glycogen availability under space flight and microgravity conditions. This area could finally be studied.

Our new method of ultrasound-guided muscle biopsy developed ad hoc for this study has minimal surgical risks, leaves minimal scarring, and has a fast recovery (~24 hours). It has allowed us to have access to elite athletes, who have avoided skeletal muscle biopsies in the past due to the invasive techniques, scars, and prolonged recovery. Our muscle biopsy technique may allow new studies with this population of endurance athletes.

Conclusion

Pre- and post-exercise ultrasound scans using MuscleSound technology were highly correlated with histochemical glycogen assessment through muscle biopsy. Changes in glycogen content from pre- and post-exercise were also highly correlated between MuscleSound technology and muscle biopsy histochemical analysis. These results show that the use of high-frequency ultrasound through MuscleSound technology is an accurate and reliable method to measure skeletal muscle glycogen in a practical, rapid, and noninvasive way. We believe this methodology can be of great importance, opening new doors for research and applicability in the field of sports medicine, sports performance, and nutrition. This may help improve our ability to care for medical conditions (eg, glycogen storage disease, diabetes, obesity) and improve nutritional assessment in critically injured patients.

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Conflict of Interest Statement

John C Hill, DO, FAAFP, FACSM, discloses conflicts of interest with MuscleSound, LLC and Newton Shoes. Iñigo San Millan discloses a conflict of interest with MuscleSound, LLC. This study was supported by an unrestricted grant from MuscleSound, LLC.

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