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Microvascular Perfusion and Intramuscular Temperature of the Calf During Cooling

Noelle M Selkow, PhD, ATC¹, Carly Day, MD², Zhenqi Liu, MD³, Joseph M Hart, PhD, ATC³, Jay Hertel, PhD, ATC, FNATA, FASCM³, and Susan A Saliba, PhD, MPT, ATC³

¹Illinois State University, School of Kinesiology and Recreation, Normal, IL

²University of Utah, Salt Lake City, UT

³University of Virginia, Charlottesville, VA

Abstract

Purpose—To examine how the microvasculature of the gastrocnemius changed after a cryotherapy intervention based on subcutaneous tissue thickness. A secondary purpose was to compare intramuscular temperature change to subcutaneous tissue thickness.

Methods—This was a single-blinded crossover study; each subject received both conditions (cryotherapy or sham). Subjects had baseline measurements of blood flow, blood volume, and intramuscular temperature recorded at 1cm into the muscle belly of the medial gastrocnemius. The randomized condition was applied for 10, 25, 40, or 60min depending on subcutaneous tissue thickness. Immediate post treatment microvascular measures were taken. After a designated rewarm period, again based on subcutaneous tissue thickness, measurements were retaken. At least 48 hours separated the two conditions.

Results—There were significant condition by time interactions for blood flow ($p=0.01$), blood volume ($p=0.022$), and intramuscular temperature ($p<0.001$). For blood flow and volume, the cryotherapy condition maintained baseline levels, while the sham condition increased at immediate-post treatment and rewarm. For intramuscular temperature, the cryotherapy condition caused a decrease in intramuscular temperature from baseline compared to no change in the sham condition from baseline. Intramuscular temperature change was significantly correlated to subcutaneous tissue thickness ($r=.49$; $p=0.05$).

Conclusions—Cryotherapy did not decrease blood flow and blood volume from resting levels, even though the intramuscular temperature decreased. An intramuscular change of 7–9°C may not be cold enough to cause local vasoconstriction.

Keywords

Contrast-enhanced ultrasound; cryotherapy; skeletal muscle; thermocouple

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Corresponding Author: Noelle M Selkow, PhD, ATC¹, Illinois State University, School of Kinesiology and Recreation, Campus Box 5120, Normal, IL 61761, 309-438-1875 (o); 309-438-5559 (f); nselkow@ilstu.edu.

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Introduction

Cryotherapy is the use of therapeutic cooling to treat injury, particularly acute musculoskeletal trauma. After injury, histamine initiates vasodilation (13, 34) resulting in pain, swelling, and secondary cell death with increased vascular permeability (29). Within sports medicine, RICE (Rest, Ice, Compression, and Elevation) is a commonly used strategy to combat the initial effects of injury and inflammation. Ice has been proposed to attenuate secondary hypoxia and edema (29, 45), decrease blood flow to the cooled area (30), and decrease pain (8, 38). The application of ice is thought to provoke clotting and control acute inflammatory responses by vasoconstriction of blood vessels in the injured area (16, 23), as well as attenuate secondary hypoxia by slowing down cell metabolism (28).

Hypoxia results when death of uninjured, healthy cells occurs after initial injury due to lack of oxygen by decreased blood flow from increased blood viscosity, muscle spasm, increased extravascular pressure, hemorrhage, and the clotting cascade maintaining homeostasis (15). Hypoxia can last for minutes, or upwards of 6 hours, depending on the injury (5, 6). After this period of decreased blood flow, reperfusion occurs, however, producing free-radicals and resulting in further injury, including increased edema (33). Cryotherapy decreases metabolic demand by slowing down the need of oxygen by mitochondria (29). It is unclear what the target change in skeletal muscle temperature should be to maximize the benefits of ice. From the literature, a change 7°C was used to identify changes in treatment time across groups with differences in adipose tissue(31), but it is unknown if this change represents effective cooling or influences intramuscular perfusion.

When skeletal muscle is injured (strain, contusion), the goal would be to cool the injured area quickly and efficiently. Muscle cooling times vary based on adipose tissue thickness (31) with increased thicknesses needing more time to cool. Therefore, the typical 20-minute cooling time would not be equally effective for everyone. Although there is an assumption that cryotherapy decreases microvascular perfusion to skeletal muscle, this hypothesis has not been studied extensively, especially in human subjects. By changing perfusion of injured muscle, two outcomes may occur. The first would be that recovery from injury would occur more rapidly since hypoxia and edema are attenuated, or the second would be that recovery from injury would be delayed since oxygen and nutrient delivery are slowed. Identifying what role ice has on skeletal muscle perfusion would allow for better understanding of this modality and if increasing, decreasing, or maintaining perfusion is more beneficial during acute injury.

Contrast enhanced ultrasound (CEU) is a non-invasive method to measure microvascular volume in muscle (39). Infused microbubbles act as the contrast agent through continuous intravenous injection at a constant rate and concentration (43). Based on the principle that ultrasound does not transmit through air and media of differing densities have greater contrast, the microbubbles can be visualized with a diagnostic ultrasound imager (17). During the continuous infusion, high-energy ultrasound is used to disrupt the microbubbles. Sequential ultrasound images are collected at variable times to detect microbubble replenishment within the vasculature (39, 43). Microbubbles are smaller than a red blood cell (1, 43), with an average size of 2.5µm and concentration of $1.2 \times 10^{-9} \text{ mL}^{-1}$ (43). The outer covering of the microbubble is composed of a phospholipid bilayer and the inside is composed of air and gas (43). Several articles have been published describing the use of CEU in skeletal muscle (10, 19–23, 39, 41, 42). Within our lab, intrarater reliability was found to be .96 (blood volume) and .92 (blood flow). Interrater reliability was found to be .82 (blood volume) and .68 (blood flow). Minimal detectable change for blood volume and blood flow were calculated at 0.35 dB and 0.026 dB/sec, respectfully.

Therefore, the purpose of our study was to examine the microvascular changes in the gastrocnemius after a cryotherapy intervention based on subcutaneous tissue thickness using CEU. Our first hypothesis was that cryotherapy would decrease blood volume and blood flow immediately after the treatment was removed compared to a sham group. Our second hypothesis was that the change in intramuscular temperature would be related to subcutaneous tissue thickness. Our third hypothesis was that the change in intramuscular temperature would be related to change in microvascular perfusion.

Methods

This was a single-blinded crossover design. The clinician who performed the microvascular perfusion measurements was blinded to the condition and recording of temperature. After the study was complete, this clinician reduced the data before condition allocation was known. Then the analysis was run. Condition order was randomized and concealed in envelopes. A second clinician administered the treatment and placed a towel over the condition to maintain blinding from the clinician reducing the data. Each subject received both conditions with at least 48 hours between sessions. The independent variables were condition (cryotherapy vs. sham) and time (baseline, immediate post-treatment, and rewarm). The dependent variables were blood flow (dB/sec), blood volume (dB), and intramuscular temperature (°C) all measured 1cm subadipose into the medial gastrocnemius muscle belly.

Subjects

Nineteen healthy adults between the ages of 18–30 years old (3 Males, 16 Females, Age: 23.2 ± 3.2 years, Height: 166.0 ± 11.7 cm, Mass: 66.2 ± 15.0 kg, skinfold (skin + adipose tissue) of the medial aspect of the gastrocnemius: 22.2 ± 7.2 mm) volunteered to participate. While we would have liked more males in our study, we did not find in the literature that gender differences existed in vascular responses to cold, just skinfold thickness. One subject was in the 0–10mm group, 8 subjects in 11–20mm, 6 subjects in 21–30mm, and 2 subjects in 31–40mm. Subjects were recruited via email, fliers, and the university's health systems clinical trials website. Subjects were excluded if they had a skinfold measurement of the medial gastrocnemius greater than 40mm, lower extremity injury in the last 6 weeks, lower extremity surgery in the last year, allergy/sensitivity to cold, taking vasoactive agents, were pregnant, or had an abnormal ECG. Subjects participated in moderate physical activity at least 3 times a week for 30 min. No one was competing at the collegiate or elite level. All subjects provided written informed consent and the study was approved by the University's Institutional Review Board of Health Sciences Research (IRB-HSR #13753).

Instruments

Contrast Enhanced Ultrasound—The SONOS 7500 ultrasound machine (Philips Medical Systems, Andover, MA) was used to image microvascular perfusion. A reliability analysis was run to assess the accuracy of our lab at measuring microvascular perfusion. Intrarater reliability was $ICC_{(2,1)} = .98$ (.91–.99) and interrater reliability was $ICC_{(2,1)} = .96$ (.87–.99). ICCs were calculated from two researchers (NS and SS) analyzing CEU data from 10 subjects. Prior to the analysis, the researchers practiced analyzing CEU data for months and comparing the measurements to the known measurements. The researchers were each trained in the technique by another researcher (ZL) whom has years of experience collecting and measuring microvascular perfusion data. Each reviewer was blinded to the measurements of the other reviewer prior to the ICC calculation.

The S3 adult echo transducer was used with an imaging ratio of 1.3:3.6 MHz. A mechanical index of 1.5 was used to initially destroy all microbubbles in the region of interest. This

machine and transducer has been used previously in several studies for myocardial and skeletal muscle perfusion by one of our co-authors (4, 9, 25, 26, 40), whom is the imaging coordinator of the General Clinical Research Center. Definity® microbubbles (Bristol Myers-Squibb Imaging, North Ballerina, MA) is an FDA approved contrast agent containing octafluoropropane gas-filled albumin. A solution of 0.9 mL of contrast agent was mixed with 21 mL of saline. Infusion was set 1.5 mL/min. Once steady state was reached (~2min), intermittent imaging was recorded at pulse intervals of 1, 2, 3, 4, 5, 8, 12, 16, and 20 cardiac cycles. Three images were recorded at each pulsing interval (11).

Intramuscular Temperature—The Physitemp Thermes USB (Physitemp Instruments Inc, Clifton, NJ) was used to record temperature. We calculated reliability using interclass correlation coefficients ($ICC_{2,1}$) and validity using a Pearson's product correlation. Intrarater reliability was .91 (.68–.97) and interrater reliability was .97 (.88–.99). Validity was $r = .97$. An IT-21 thermocouple (Physitemp Instruments Inc, Clifton, NJ) was inserted 1cm into the muscle to measure temperature using a posterior approach. Once skinfold was calculated (skin + adipose tissue), 10mm was added. The thermocouple was marked with permanent marker to the depth of proper insertion for each subject. The tip of the needle, with the thermocouple inside, was inserted 5cm for each subject. The thermocouple was retracted until the mark was identified, indicating proper depth was reached. Previous research has shown the IT-21 to not be as reliable as the IT-18 in extreme temperatures of 5°C and 35°C (27), however, we used two new thermocouples and allowed the temperature to stabilize for 10 min before applying either condition. We felt confident the baseline temperatures recorded represented muscle temperature. For sanitization, the thermocouples were submerged in Cidex Plus® (Johnson and Johnson, Irvine, CA) for 20 minutes after each use. Manufacturer testing reports 99.8% of resistant *Mycobacterium tuberculosis* is destroyed during that time frame and is non-corrosive to instruments.

Procedures

The study consisted of 4 visits. For visit 1, the subjects completed health history questionnaires and had vital signs examined (blood pressure, pulse, respirations, and heart auscultation) by a physician. Skinfold of the medial gastrocnemius on the non-dominant leg was measured at the thickest part of the calf using real-time ultrasound (LOGIQ Book XP; GE Healthcare Products, Milwaukee, WI). Skin and adipose tissue were measured using the ultrasound machine over the muscle belly of the medial gastrocnemius, then doubled to be put into the categories previously mentioned by Otte *et al* (31). We chose to use ultrasound imaging instead of pinch calipers, since research from our lab found ultrasound imaging to be more reliable than pinch calipers at measuring subcutaneous/adipose tissue (34).

Visit 2 required the subject to have a metabolic blood draw to test liver and kidney function, ECG, and serum pregnancy test if female. These tests were conducted at the General Clinical Research Center (GCRC) of the university hospital. A physician reviewed the history, blood profile and ECG. If deemed healthy, the subject was allowed to begin with the intervention testing.

During Visit 3, the subject reported to the GCRC. If female, a urine pregnancy test was administered, since anywhere from 1 week to 1 month may have passed between the initial serum pregnancy test. A pregnancy test needed to be administered if more than 48 hours had passed since the last pregnancy test when microbubbles were to be administered, per protocol of the GCRC. A registered nurse inserted an IV into an antecubital vein of the dominant arm. A 3-lead ECG was used to monitor the subject's heart rhythm and was the trigger for ultrasound image capture. The subject was positioned prone and the non-dominant leg was cleaned and prepped. A physician anaesthetized the area of the medial

gastrocnemius with 1cc of lidocaine without epinephrine. Epinephrine helps to reduce bleeding, which could possibly affect vascular function. This area was marked with a $2.54\text{cm} \times 2.54\text{cm}$ square, which was the size of the transducer. A thermocouple was inserted into the middle of the area using an 18g needle, 1 cm into the medial gastrocnemius muscle, accounting for skin and adipose tissue, and covered with a translucent dressing (Figure 1). Large blood vessels were avoided. The thermocouple was marked to ensure that proper placement was achieved, taking into the account the thickness of the skin and adipose tissue thickness. During Visit 4, the thermocouple was inserted back in the same area as in Visit 3. Temperature was recorded every second throughout the entire session. After waiting 10 minutes for the intramuscular temperature to stabilize, the infusion of the contrast agent began. When the contrast agent was considered to be consistent throughout the vascularity, which took approximately 2 minutes, CEU measurements of microperfusion began. The transducer was applied over the translucent dressing to the area marked on the medial gastrocnemius and images were recorded at pulse intervals (PI) of the ECG, with three images taken at every 1 beat, 2 beats, 3 beats, 4 beats, 5 beats, 8 beats, 12 beats, 16 beats, and 20 beats, resulting in 27 images (Figure 2). The CEU measurements imaged to a maximum depth of 6 cm below the skin. This allowed more than enough depth for the region of interest (ROI) to be measured at the depth of the thermocouple. After the images were recorded, approximately 5 minutes later, the randomized condition was applied.

Interventions—For the cryotherapy condition, a 750g crushed ice bag was placed over the medial gastrocnemius. The time was dependent on the amount of subcutaneous tissue for the temperature to reach a 7°C decrease from baseline (Table 1) (14, 31). For the sham condition, a 750g bag of candy corn was applied for the same duration as the cryotherapy condition. After the intervention was removed, microvascular perfusion measurements were taken again using CEU. After a designated rewarm period, based on research from Jutte *et al.* (14), microvascular perfusion measurements were taken again using CEU. The rewarm period was projected to return intramuscular temperature within 2°C of baseline (Table 1). After the CEU measurement, the subject's vital signs and ECG were monitored for 30 minutes before being discharged. At least 48 hours passed before the subject started Visit 4, which was the same as Visit 3, except they had the opposite condition.

Data Analysis—Using QLAB (Philips Healthcare, Andover, MA), replenishment kinetics were calculated for blood volume and blood flow velocity. Measurements begin as the microbubbles begin to accumulate in the ultrasound beam (E), or ROI again, which is based on velocity (v) and distance (d) the microbubbles need to travel using the following equation: $d=vt$ (43), where t is the pulsing interval (PI) of the ultrasound (43), or when the ultrasound captures a picture of the ROI. The ROI was manually circled at the depth of the thermocouple, so microvascular perfusion and temperature could be compared at the same muscle depth. The circle was drawn on one image and overlaid on the remaining 26 images of a CEU measurement, so the same area on all images was used in the calculation. The area of the circle was recorded, so the same sized circle would be drawn on subsequent CEU images from the other time points. Video intensity (VI) is proportional to d/E due to its proportion to microbubble concentration at any PI (41). VI is a measurement of sound, so it is recorded in dB. If d is substituted in the above equation, VI is proportional to vt/E (43). VI will increase with t when v is held constant until the microbubbles have filled E at a specific PI called T , resulting in the equation $E=vT$ (22). VI will eventually become constant, plateau (A), when T is exceeded. This indicates that there is sufficient microbubbles within the microcirculation of the ROI. A is proportional to the cross-sectional area of microvessels within E (43). The equations above are based on VI and PI being uniform, however, E and destruction of the microbubbles are probably not uniform and microbubbles are not flat (43). Therefore, the relationship between VI and PI is best described using an exponential

function; $y=A(1-\exp^{-\beta t})$ (43). y equals VI at PI t , VI plateau (blood volume) is A , and β is the rate of rise in VI (blood flow velocity) (43). Blood flow (f) within the microvascular structures can be expressed as $f=A\beta$ (43).

To quantify microvascular blood flow, enhanced signals from the larger blood vessels are subtracted from the microvascular contribution (39). Larger vessels are distinguished from the microvascular structures due to the short delay in image enhancement (43). Microbubbles remain within the vasculature unlike contrast agents used in MRI that diffuse into the interstitium. This allows for microvascular perfusion measurements to be calculated (37). A main limitation to replenishment kinetics described above is the information is calculated from a single plane, not 3-D (32).

Statistical Analysis

We performed a sample size estimation using blood flow and blood volume as the dependent measurements from a previous study (26). Eighteen subjects were needed to find significant differences with power set at 80% and alpha set at 0.05. We performed separate 2×3 repeated measures ANOVAs for all dependent variables. Pearson's Product Correlation compared subcutaneous tissue thickness to change in intramuscular temperature immediately post treatment in the cryotherapy condition, as well as temperature change to changes in microvascular perfusion. Alpha was set *a priori* at $\alpha=0.05$. SPSS 17 (Chicago, IL) was used for statistical analysis.

Results

There was a significant condition by time interaction for blood volume ($p=0.022$), blood flow ($p=0.01$), and intramuscular temperature ($p<0.001$). Means and SD are presented in Table 2. There was a significant correlation ($r=.49$) between subcutaneous tissue thickness and change in intramuscular temperature immediate post treatment ($p=0.05$) for the cryotherapy condition. Significant correlations were also found for change in temperature during the rewarm period and change in blood volume at rewarm ($r=.53$, $p=.033$) and change in blood flow at rewarm ($r=.56$, $p=.025$) for cryotherapy.

Discussion

Microvascular perfusion of the gastrocnemius did not decrease from baseline when cryotherapy was applied, even though intramuscular temperature decreased. Our data is consistent with research using plethysmography (3, 12). The increase in perfusion seen in the sham condition may have been due to microtrauma associated with the insertion of the thermocouple. Cryotherapy may attenuate inflammation from the microtrauma possibly explaining our significant differences between the cryotherapy and sham conditions immediately after the intervention was removed, as well as after the rewarm period. While baseline blood flow and volume measurements seem to be different between the conditions, they were not statistically different.

We were surprised that cryotherapy did not decrease skeletal muscle blood flow from baseline. However, the stability of the baseline values with ice application may have a protective function. Decreases in the resting, baseline blood flow might result in hypoxic conditions, resulting in death to healthy tissues (29). Furthermore, since the muscle and deeper tissues lack thermal receptors, the mechanism associated with changes in skeletal muscle tissue perfusion with thermal gradients would likely be from a reflexive mechanism from the skin (2, 46). Another explanation for not observing a change in local microvascular perfusion is that cryotherapy does not seem to alter arteriole diameter, at least in animal

models. Smith *et al.*(35) reported no changes in arteriole diameter following repeated 20 min ice application over 18 hours, resulting in no microvascular changes.

While there have been no reported studies using CEU with ice application, several studies have used the same CEU method and analysis for changes in microvascular perfusion for peripheral artery disease and diabetes (7, 24, 25, 44). Changes have been detected between healthy and diseased populations using this method, verifying that changes in perfusion can be detected by CEU and these changes help to determine the severity of disease and disease process.

The question remains of what, if any, intramuscular temperature change is beneficial and if that change affects blood flow. Knowledge of this would help clinicians appropriately time ice application and maximize the benefits of cryotherapy during the acute phase of inflammation. A decrease in blood flow may result in decreased bleeding and intramuscular hematoma (12), however at what intramuscular temperature this occurs at is uncertain and if this is important. We know from our study that intramuscular temperature decreased by about 7°C with no decrease in blood flow. It is unclear if this decrease in intramuscular temperature would be effective in injury recovery and how blood flow would change when ice is applied to injured tissue.

Future research should focus on the effect of cryotherapy to an inflamed or exercised condition, since both of these conditions have been shown to increase blood flow substantially. Measuring blood flow changes when cryotherapy is applied in these conditions would help determine if decreases in blood flow are occurring. If it does, we can further examine at what intramuscular temperature blood flow decreases and if this is beneficial during the inflammatory process. A longer rest period by the subject should also be implemented before beginning CEU measurements. This will allow microvascular perfusion to stabilize and result in more accurate measurements.

In conclusion, this is the first study to examine the effect of a therapeutic agent on microvascular perfusion using CEU. Cryotherapy did not decrease blood flow below baseline levels in healthy tissue indicating that an intramuscular change of 7–9°C may not be cold enough to cause local vasoconstriction.

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Figure 1. Thermocouple Insertion

A=Connection to Physitemp USB

B=18g needle used to insert thermocouple

C=Thermocouple secured to area by translucent dressing

Figure 2. Region of Interest and Replenishment Kinetics

The image represents a transverse view of the medial gastrocnemius. The white circle is the region of interest where the perfusion measurements were captured. This spot was chosen as it is close to the depth of the thermocouple and avoids large vessels (bright white areas), since we were interested in the blood flow of the capillaries only. The graph represents replenishment kinetics and the equation: $y=A(1-\exp^{-\beta t})$.

Table 1

Condition application and rewarm times based on subcutaneous tissue

Subcutaneous Fat Thickness (mm)	Application time for condition (min)	Rewarm period after condition removed (min)
0–10	10	30
11–20	25	60
21–30	40	90
31–40	60	140

Table 2

Mean $\pm$ SD values for blood volume, blood flow, and intramuscular temperature within and between conditions

	Blood Volume (dB)			Blood Flow (dB/sec)			Intramuscular Temp ($^{\circ}$ C)		
	Base	IP	RW	Base	IP	RW	Base	IP	RW
Ice	9.47 $\pm$.92	7.55 $\pm$.75	7.98 $\pm$.83	3.14 $\pm$.21	2.79 $\pm$.28	3.01 $\pm$.27	36.39 $\pm$ 1.77	26.99 $\pm$ 5.11 ^{*F}	29.79 $\pm$ 2.05 ^{*F}
Sham	7.32 $\pm$.81	10.42 $\pm$.98 ^{*F}	11.43 $\pm$.91 ^{*F}	2.59 $\pm$.23	3.43 $\pm$.25 [*]	4.10 $\pm$.28 ^{*#F}	36.64 $\pm$.70	36.09 $\pm$ 1.39	35.80 $\pm$ 1.44

Base=Baseline, IP=Immediate Post-Condition, RW=Rewarm

^F =significantly different between conditions p<.05

^{*} significantly different from baseline within condition p<.05

[#] significantly different from IP within condition p<.05