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Cryotherapy-Induced Persistent Vasoconstriction After Cutaneous Cooling: Hysteresis Between Skin Temperature and Blood Perfusion

The goal of this study was to investigate the persistence of cold-induced vasoconstriction following cessation of active skin-surface cooling. This study demonstrates a hysteresis effect that develops between skin temperature and blood perfusion during the cooling and subsequent rewarming period. An Arctic Ice cryotherapy unit (CTU) was applied to the knee region of six healthy subjects for 60 min of active cooling followed by 120 min of passive rewarming. Multiple laser Doppler flowmetry perfusion probes were used to measure skin blood flow (expressed as cutaneous vascular conductance (CVC)). Skin surface cooling produced a significant reduction in CVC ($P < 0.001$) that persisted throughout the duration of the rewarming period. In addition, there was a hysteresis effect between CVC and skin temperature during the cooling and subsequent rewarming cycle ($P < 0.01$). Mixed model regression (MMR) showed a significant difference in the slopes of the CVC–skin temperature curves during cooling and rewarming ($P < 0.001$). Piecewise regression was used to investigate the temperature thresholds for acceleration of CVC during the cooling and rewarming periods. The two thresholds were shown to be significantly different ($P = 0.003$). The results show that localized cooling causes significant vasoconstriction that continues beyond the active cooling period despite skin temperatures returning toward baseline values. The significant and persistent reduction in skin perfusion may contribute to nonfreezing cold injury (NFCI) associated with cryotherapy. [DOI: 10.1115/1.4032126]

Keywords: cryotherapy, cold-induced vasoconstriction, cooling and warming hysteresis, skin blood flow

Introduction

Localized cooling (cryotherapy) of soft tissue is commonly recommended following orthopedic surgery and sports injuries to reduce pain, swelling, inflammation, bleeding, and secondary hypoxic injury. Cryotherapy may be used in conjunction with cryokinetics by physical therapists to reduce the sensation of pain and to allow early mobilization [1,2]. Although cryotherapy has been adapted widely, protocols largely have been derived empirically, and many diverse operational methods are recommended [3].

An important aspect of cryotherapy that is not widely documented and acknowledged is the strong effect of lowered skin temperature on reducing local tissue perfusion and mechanisms by which this phenomenon may lead to the occurrence of NFCI. Thus, the authors have undertaken an extensive series of experiments to measure the dose dependency and time progression of the vasomotor response to low temperature exposure. Key results from these studies are reported herein.

Prolonged cold-induced vasoconstriction may result in tissue ischemia leading to NFCI [4–6]. Furthermore, re-establishing tissue blood flow following a sufficiently prolonged period of tissue ischemia may result in reperfusion injury [7]. There are sundry reports in the cryotherapy literature of ischemic related injuries ranging from ice burn [8] to full thickness skin necrosis [9,10] and

nerve damage [11–13]. Understanding the effect of cold application on tissue perfusion is essential in developing treatment protocols that could reduce the risk of developing tissue injury as a result of cryotherapy. The current study aims at improving the understanding of vascular flow alterations during and after termination of a cryotherapy episode.

Localized cooling of nonglabrous skin results in vasoconstriction through activation of vasoactive pathways [14–18]. There are indications that the cutaneous vascular response to local cooling occurs in phases [15]. To the best of our knowledge, a similar understanding does not exist regarding the vasomotor response during the passive rewarming period following active cryotherapy cooling.

Hysteresis describes a process for a system wherein the current state is dependent on its history and initial value. For a system undergoing a process involving hysteresis, the values of dependent (output) properties do not map directly from the values of the independent (input) properties. In the present consideration, the system consists of tissue undergoing a cooling process with the surface temperature being the independent property and the cutaneous blood perfusion being the dependent property. Hysteresis may be applied to identify and describe irreversible aspects of a process. Hysteretic processes occur throughout nature [19] and there are many examples of hysteresis in biology [20].

The current work illustrates the hysteresis phenomenon associated with the vasoactive processes elicited by temperature alterations in tissue that govern the extent to which blood perfusion may retrace its thermodynamic state pathway as temperature falls and rises during a cryotherapy cycle. We have observed that it is not possible to use simple temperature manipulations to cause the variation of blood perfusion to follow the same temperature dependent history during warming as occurred during cooling,

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Manuscript received September 2, 2015; final manuscript received November 20, 2015; published online January 29, 2016. Assoc. Editor: Ram Devireddy.

resulting in a hysteresis loop when perfusion is plotted as a function of temperature for a completed cryotherapy application cycle. The consequence is a direct effect on the history of blood supplied to tissue in a targeted region of therapy and the potential risk factors for ischemic induced injury.

Despite the widespread use of cryotherapy and the occurrence of related injuries in some patients, the relationship between skin temperature and tissue perfusion during the cooling and subsequent rewarming remains relatively unknown. Furthermore, the sequential phases of the vascular response to localized cooling are not well characterized. Therefore, a primary goal of this study was to investigate the relationship between skin temperature and CVC during and following localized cooling of nonglabrous skin with a commercially available CTU. Also, the existence of a threshold temperature defining a change in the slope of CVC/temperature curve between the early and the later stages of cooling and rewarming was evaluated. We hypothesize that the foregoing vasomotor phenomena drive a hysteresis effect between CVC and skin temperature during the cooling and rewarming phases of cryotherapy, in the absence of artificial external stimuli.

Methods

Ethical Approval and Subjects. This study was approved by the Institutional Review Board of The University of Texas at Austin prior to the start of experiments. All subjects provided voluntary written informed consent to participate.

The experiments were performed on six healthy nonsmokers (three male) with an average age ($\pm$ SE) of 22.5 ± 1.4 years and body mass index (BMI) ($\pm$ SE) of $23.4 \pm 1.6 \text{ kg/m}^2$. Five subjects (two male) were enrolled and underwent control experiments where skin-temperature water instead of ice-cold water was used to perfuse the cooling pad. The goal of these experiments was to isolate any possible contribution to the outcome due to the instrumentation procedure or flow of water through the pad. The average age of control subjects was ($\pm$ SE) 32.2 ± 4.5 years with an average BMI of ($\pm$ SE) $23.3 \pm 1.9 \text{ kg/m}^2$. All subjects were free from any known cardiovascular, metabolic, or neurological disorders and were not taking any medication. None of the subjects reported a history of cryotherapy or other form of cold exposure in the lower extremities for at least a year prior to the experiment and had no history of knee injury for at least three weeks prior to enrolling in the study. Each subject participated in a single identical trial.

Instrumentation and Measurements. All experiments were performed in the right knee region with the subject positioned in a semi-recumbent posture in a laboratory environment with room temperature ($\pm$ SE) of $21.8 \pm 0.4^\circ\text{C}$ and relative humidity of 50%. Prior to the start of data collection, the right knee of each subject was instrumented with three laser Doppler flowmetry probes (two VP1T/7 and one VP1-HP in conjunction with MoorVMS-LDF2 and MoorVMS-LDF1-HP monitors, respectively; Moor Instruments, Millway, Axminster, Devon, UK) to provide a continuous index of cutaneous blood flow. The average interrogation depth of MoorVMS-LDF2 and MoorVMS-LDF1-HP were about 0.5 and 2 mm, respectively [21]. Several copper-constantan thermocouples (Omega Engineering, Stamford, CT) were placed at multiple sites under the cooling pad to monitor skin temperature. Perfusion measurements were obtained at three different locations: proximolateral, distolateral and proximomedial to the knee joint. Figure 1 presents an image of instrumentation prior to the application of the thermal barrier wrap. This figure also shows the general location of the various probes in the active treatment area.

Following instrumentation, the measurement area was carefully wrapped with a single layer of loose all-cotton elastic (ACE) bandage to provide a thermal barrier between the skin surface and the cooling pad. Then a cooling pad (Arctic Ice universal pad;

Fig. 1 Application of instrumentation at a measurement site. P1, P2, and P3 are the three perfusion probes. T shows the locations of thermocouples. This site will be covered with the cooling pad.

Pain Management Technologies, Akron, OH) was applied overlying the instrumented area, and fixed in place using another layer of Ace bandage. An Arctic Ice CTU (Pain Management Technologies, Akron, OH) was used to deliver cold water through the cooling pad applied to the subject's right knee following the manufacturer's recommendation (see below for more detail). An earlier set of experiments showed no significant difference in the extent of reduction in tissue perfusion using different CTUs [22]. The Arctic Ice CTU was used since its pad had a greater surface temperature uniformity compared to other CTUs [23]. Intermittent blood pressure measurements were obtained every 15 min by auscultation of the brachial artery with an electrophygmomanometer (SunTech, Raleigh, NC), and mean arterial blood pressure was calculated as one-third of pulse pressure plus diastolic pressure.

Additional thermocouples were placed on the anterior surface of the left shin, right thigh, right arm, and chest for monitoring of mean skin temperature using the method of Ramanathan [24]. Core temperature was measured via a type T bead thermocouple embedded in the tip of a single use, silicone ear plug placed at the proximity of the tympanic membrane and occluding the ear canal from external effects [25–27]. In addition to the laser-Doppler flowmetry probes located at the treatment site, two additional probes were located at control sites not exposed to the cooling; one on the dorsal surface of the right foot distal to the treatment area, and another on the lateral surface of the left knee proximal to the joint.

Experimental Procedure. Subjects remained in a semi-recumbent position for the duration of the experiment and were asked to refrain from moving their lower extremities as much as possible to avoid causing artifacts in the laser Doppler signals. Subjects became acclimated to room temperature during 1 hr of instrumentation prior to the start of data collection. A blanket was provided if requested to prevent vasoconstriction due to exposure to room temperature air. Following instrumentation (including placement of the cooling pad) there was a 30-min period of baseline data collection with no active change in skin temperature. Next was one hour of active skin-surface cooling at the treatment site with the CTU circulating water at an average temperature of ($\pm$ SE) $2.4^\circ\text{C} \pm 0.5$ through the pad with the exception of the control experiments where the water temperature was set to that of average skin temperature, measured during the baseline period, at the treatment site. As mentioned earlier the recommendations for the duration of cryotherapy application are highly varied ranging from minutes to days. In this context, the duration of active cooling was set to 60 min to produce a significant change in CVC while keeping the risk of vasoconstriction side effects low and the total experimental duration acceptably short for subjects. At the end of the cooling phase the CTU was turned off while data

collection continued for an additional 2 hrs with all instrumentation undisturbed. During this passive rewarming phase of the cycle heat propagation from the deeper tissue and ambient environment increased the tissue temperature. Table 1 shows the experimental parameter ranges.

Data Extraction/Analysis. Hemodynamic and thermal data were collected on a data acquisition system (National Instruments (NI) input modules 9205, and 9213, National Instruments, Austin, TX) and subsequently transferred to a laboratory computer via an NI DAQ 9174 (National Instruments, Austin, TX). All perfusion data were collected with a sampling rate of 12 Hz. Temperature data was sampled at the rate of 1 Hz. Three laser Doppler flowmetry perfusion probes were used under the cooling pad in each experiment to measure skin perfusion. VP1T/7 perfusion probes that feature a thermistor integral to the sensor tip were also used to measure skin temperature and blood perfusion at a single identical site. There was no significant difference among the average perfusion values measured by VP1T/7 and VP1-HP probes. Therefore all perfusion and CVC data presented herein is based on the aggregate averages of VP1T/7 probes. All reported skin temperatures are the average values obtained with the VP1T/7 probes. In order to account for potential blood pressure changes during the protocol, all flow data is reported as CVC as calculated by $CVC = \text{laser Doppler flux units/mean arterial pressure}$.

Data from the cooling and rewarming periods were divided into 1-min segments from the start of each phase of a trial. Baseline data was calculated as the median value for perfusion and CVC during the last 5 min of that period. Likewise, median values for perfusion and CVC were determined during each of the 1 min data segments during cooling and rewarming. The same procedure was used to extract temperature data with the exception that the median was replaced with mean to calculate a representative value for each temporal data segment. This data was used to study CVC/skin temperature hysteresis (for visualization and area calculation, see below). For all other applications, data was downsampled to 1 value/5 min. Data extraction was performed in MATLAB (MATLAB 7.10, The MathWorks Inc., Natick, MA, 2010) in a semiautomated fashion with the only input from the researcher being the designation of the starting and ending times of active cooling.

CVC values were normalized to the baseline period and calculated as percent change from that state. The minimum CVC measured during the cold exposure was identified and compared to the baseline value using a one-sample *t*-test in MATLAB.

Persistence of Vasoconstriction During the Rewarming Period. A case-control study using the Dunnett's test was performed to compare CVC and skin temperature from the first 5 min of rewarming with future time points. Additionally, repeated measure analysis of variance (rmANOVA) and Freidman tests were used to compare CVC values from the first 5 min of rewarming with that for all other subsequent time points. Bonferroni correction and false discovery rate (FDR) control were used to adjust the *p*-value for multiple comparison tests. The modified Z-score method was used to identify possible outliers [28]. The Shapiro–Wilk's test was used to assess the assumption of data normality at each time point and the Maulchly's test to assess the

assumption of data sphericity. MMR was used to evaluate the possibility of an increasing trend in CVC during rewarming and its statistical significance. MMR was applied for this purpose rather than linear regression due to the repeated measure nature of the data, which could potentially violate the assumption of independence in linear regression. SPSS (IBM SPSS Version 21.0 for Windows; Armonk, NY: IBM Corp) was used for all statistical analysis with the exception of the one sample *t*-test and the Dunnett's test [29] which were performed in MATLAB.

Sequential Stages of Cutaneous Vasomotor Response. Our observation of the characteristics of the tissue perfusion history, derived from conducting and evaluating hundreds of cryotherapy trials, indicates that the rate of change in perfusion is governed by a two-step process with an identifiable intermediate breakpoint. Segmented regression was used to investigate the slope of the CVC/temperature curve during the early and late phases of cooling and passive rewarming to find the threshold temperature whereby the curve gradient undergoes a significant discontinuity. The perfusion and temperature data from each of the perfusion probes was fit with a piecewise regression model in SPSS after selecting a candidate threshold value based on visual observation of data. The threshold value then was changed incrementally, and the process was repeated. The threshold value that offered the smallest residuals was selected for each perfusion data set. The threshold values identified for both the cooling and rewarming periods were compared using a paired *t*-test. The time point associated with the threshold value was also extracted.

Hysteresis. MMR was applied using SPSS to fit the magnitude of CVC to the skin temperature during both the cooling and passive rewarming periods. The result was an empirical quadratic equation with a temperature² term added to the standard linear regression expression (intercept + temperature) to accommodate the nonlinear behavior of the data. The temperature and temperature² were used as fixed effects to determine the overall trend and as random effect to calculate the variation amongst different subjects. A flag variable was introduced to differentiate between the cooling and the rewarming portions of the experimental cycle. Finally, two interaction terms (between the flag variable and the temperature and the temperature²) were added to verify the significance of the differential dependence of CVC on skin temperature during cooling and rewarming. The quadratic equation used as follows:

$$\begin{aligned} CVC_{ij} &= \beta_{0j} + \beta_{1j}T_{ij} + \beta_{2j}T_{ij}^2 + k + k^*T + k^*T^2 + e_{ij} \\ \beta_{0j} &= \gamma_{00} + u_{0j} \\ \beta_{1j} &= \gamma_{10} + u_{1j} \\ \beta_{20} &= \gamma_{20} + u_{2j} \end{aligned} \quad (1)$$

where *T* stands for temperature; *k* is the flag that defines if each data point is part of the cooling or rewarming periods; γ_{00} , γ_{10} , and γ_{20} are the fixed effects coefficients; u_{0j} , u_{1j} , and u_{2j} are the random effects coefficients; *k*, k^*T , and k^*T^2 are the interaction terms; and e_{ij} is the residual error term. The subscript *j* denotes serial data points. The likelihood ratio test for random effects was used to validate the significance of

Table 1 Summary of experiments

Durations (±SE) (min)		Baseline values (±SE)		Minimum achieved (±SE)	
Baseline	30.1 ± 0.2	Temperature (°C)	31.4 ± 0.4	Temperature (°C)	16.6 ± 0.3
Cooling	60.1 ± 0.2	CVC	Absolute 0.42 ± 0.09	CVC	Absolute 0.09 ± 0.02
Rewarming	123.7 ± 4.7		Relative 100%		Relative 26% ± 3.2

Note: Parameters by which experimental protocols were defined, including baseline, cooling, and rewarming durations; average CVC and skin temperature values during the last 5 min of the baseline period, minimum values achieved after 1 hr of cold exposure. The CVC is presented both relative to baseline and in absolute format. Standard errors of means for all measured values are presented.

different terms in the regression model. The normal quantile–quantile (Q–Q) plot was used to investigate normality of residuals as a diagnostic measure to evaluate the representative accuracy of the MMR model. Sample size calculations using G*POWER version 3.1 [30,31] showed that this study carried a power of 0.84 for $n=6$ when looking at the absolute values of CVC and a power of 0.99 for $n=3$ when looking at the normalized values of CVC. Significance was accepted at $P < 0.05$.

Estimation of Temperature Change With Depth Into Tissue. The bioheat module in COMSOL MULTIPHYSICS version 4.3b was used to simulate propagation of the temperature field into the tissue during the cryotherapy cycle. A 2D geometry was applied consisting of epidermis, dermis, subcutaneous adipose tissue, and muscle with the thickness of 100 μm , 1 mm, 4 mm, and 3 cm, respectively. Tissue properties were set based on the values reported in the literature [32–34]. Initial values for tissue temperatures were calculated for the steady-state tissue temperature during exposure to ambient conditions. For the cryotherapy cycle, the surface temperature was set to the experimentally measured values shown in Fig. 2(a) for the active cooling and passive rewarming periods. The bioheat module embodies the Pennes' equation [35] that includes the thermal effect of internal convection of blood if perfused through tissue and distributed metabolic heat generation. Both the metabolic generation and perfusion terms in the Pennes' equation were assumed to be temperature dependent. A Q10 rate coefficient was applied to simulate the temperature dependence of metabolism, defined as the increment in the rate of reaction for each 10 $^{\circ}\text{C}$ change in temperature [36]. Q10 takes a value of 2–3 for chemical reactions. For the purpose of this paper, it was set at the mid-range value of 2.5 [37]. To evaluate the temperature dependence of perfusion, the MATLAB curve fitting tool was used to evaluate the relationship between temperature and perfusion during cooling and rewarming based on the data in Fig. 2(a) using an exponential fitting function. The COMSOL model was used to predict the penetration of the temperature field through the tissue during the surface cooling and rewarming cycle.

Results

For the cooling experiments, baseline skin temperature ($\pm\text{SE}$) was $31.4^{\circ}\text{C} \pm 0.4$ and reached an average minimum of $16.6^{\circ}\text{C} \pm 0.3$ at the completion of 1 hr of active cooling. Cooling had no effect on tympanic and average skin temperature. At the end of cooling, CVC was reduced significantly from its baseline value ($P < 0.001$). For the control experiments, the skin temperature ($\pm\text{SE}$) was at $31.0^{\circ}\text{C} \pm 0.6$ at end of baseline period and increased to $32.7^{\circ}\text{C} \pm 0.6$ by the end of active water flow period which lasted for ($\pm\text{SE}$) 62.2 ± 1.6 min. The average temperature

of water for the control experiments was ($\pm\text{SE}$) $30.2^{\circ}\text{C} \pm 0.3$. Exemplar data sets presented in Fig. 2 show changes in CVC and skin temperature during an experiment with ice-cold (2(a)) or skin-temperature (2(b)) water with flow lasting for 60 min. The surface skin temperature falls by $\sim 15^{\circ}\text{C}$ during cooling and increases by about 10°C during the rewarming period (Fig. 2(a)). In contrast, the CVC drops by more than 75% of baseline by the end of cooling and remains depressed for the duration of the passive rewarming period. In the exemplar data plot presented in Fig. 2(b), perfusion appears to increase during the active water flow with no specific trend in perfusion during the rewarming period. This hysteresis between CVC and skin temperature is described in more detail below. CVC measurements at control sites do not decrease during cooling at the treatment site. In the control experiment, the perfusion increases during the active water flow period by ($\pm\text{SE}$) $27.8\% \pm 13.7\%$ in contrast to cooling experiments where perfusion drops as a result of cold exposure.

Persistence of Vasoconstriction During the Rewarming Period. Since there is no single statistical test that is optimal for analyzing this data set, three alternate tests were applied to assure a strong basis for interpretation. Our goal was to determine when during the passive rewarming period a significant increase in perfusion takes place. In this context, the Dunnett's test is a suitable tool since it compares perfusion at each time interval to the control group defined as perfusion at the termination of cooling. However, given the nature of the data, the assumption of independence required for this test is violated. For that reason rmANOVA is a more suitable test, but since it compares all possible pairings between different groups, it is still not a perfect fit for this application. MMR is possibly the most suitable tool amongst these three tests, but it only shows a general trend in data as opposed to determining a specific time at which a significant change in perfusion occurs. Thus, in order to better take advantage of the collective benefits of all three tests, the results from each are reported. A case-control study applying the Dunnett's test was used to compare the changes in CVC and skin temperature during rewarming. The results show that even though temperature had a significant increase starting at 15 min into the rewarming period ($P < 0.001$), the CVC values showed no significant increase throughout the entirety of rewarming ($P > 0.05$), with the exception that CVC at 120 min was significantly higher than at 5 min ($P = 0.035$).

The modified Z-score test found no outliers in data at any of the time points. The Shapiro–Wilk's test showed that CVC data at each time point was normally distributed ($P > 0.05$). Given the sample size of 6 and the low power of normality tests for such small sample sizes [38] both rmANOVA and its nonparametric counterpart, the Friedman test, were performed. SPSS was unable

Fig. 2 (a) Percent change compared to baseline values for CVC (top panel) and skin temperature (bottom panel) for a sample experiment. The protocol followed 30 min of baseline, 60 min of active cooling, and 2 hrs of passive rewarming. **(b)** Percent change in CVC from baseline from a control experiment. The duration of active water flow through the cooling pad is marked on the plot.

to calculate χ^2 statistics and significance for the Mauchly's test, but since the estimation of ε was less than 0.75, the Greenhouse–Geisser correction was used to assess the significance of rmANOVA. Considering rmANOVA was statistically significant ($P=0.013$), a multiple comparison test was performed. Table 2 presents the results of multiple comparison tests with Bonferroni correction and FDR control, and the Friedman test. The tests with Bonferroni correction determined no significant increase in CVC during rewarming, whereas the FDR control method indicated a significant increase only at 120 min ($P=0.02$), and the Friedman test showed a significant increase only at 110 and 120 min ($P=0.048$ and 0.040, respectively).

An MMR study of the CVC rewarming data at 60, 75, 90, 105, and 120 min showed that the temporal slope of a fitted line was significantly larger than 0 ($P < 0.05$) only at 90 min and later and was insignificant earlier ($P \geq 0.05$).

Sequential Stages of Cutaneous Vasomotor Response. The threshold value defines the temperature/time point where a smaller slope in CVC/temperature transitions to a significantly faster rate of change. The average threshold temperature value during cooling and rewarming ($\pm$ SD) was 22.5°C (± 3.2) and 25.8°C (± 2.1), respectively. On average, it took 11.1 (± 6.2) and 67.9 (± 17.7) min to reach the slope breakpoint during the cooling and rewarming periods, respectively. The paired t -test showed a significant difference ($P=0.003$) between the threshold temperature values during cooling and rewarming.

Temperature/CVC Hysteresis. The persistence of vasoconstriction results in a hysteresis effect between CVC and skin temperature during the cooling and rewarming cycle. Figure 3 shows temperature and CVC values for baseline, the end of cooling, and at selected time points during rewarming. The temperature increase starts immediately upon cessation of cooling, whereas CVC continues to drop during the first 10 min of rewarming followed by a very slow increase. Eventually, CVC increases by only about 30% after 120 min of rewarming, while temperature recovers about 70% of the cooling drop. This hysteresis behavior is seen explicitly in Fig. 4 wherein CVC is plotted as a continuous function of the skin temperature during cooling and rewarming for a single trial (a) and for the average of six trials (b). Arrows mark the process direction during cooling and rewarming. During the baseline period, the skin temperature follows a mild increase due to the insulating effect of covering the treatment area with the ACE bandage and the cooling pad causing a local accumulation of heat. This temperature increase causes a temporary elevation in CVC in comparison to the initial baseline state, so that the normalized CVC is larger than 100% at the start of cooling.

The hysteresis area of the space state diagram is a direct measure of the extent of persistent suppression of local blood flow in response to the application of cooling. This area was calculated for each experiment, and the average determined to be

Table 2 Result of rmANOVA and Friedman tests

	10–100 min	110 min	120 min
rmANOVA with Bonferroni adjustment	$P > 0.05$	$P > 0.05$	$P > 0.05$
rmANOVA with adjusted Bonferroni (FDR)	$P > 0.05$	$P > 0.05$	0.021
Friedman test	$P > 0.05$	0.048	0.040

Note: Persistence of vasoconstriction in response to applied skin temperature of 16.6°C for 60 min. CVC at different time points during the rewarming period are compared to the value at the first 5 min. rmANOVA with Bonferroni and adjusted-Bonferroni (FDR method) adjustment and Friedman tests were used. The time points are defined with reference to the start of rewarming. The values presented are the results of pairwise comparison performed in SPSS. All significant P -values are expressed numerically and presented in boldface.

Fig. 3 Temperature (top panel) and absolute CVC (bottom panel) values during the last 5 min of baseline and cooling (marked as B and C, respectively) and at the end of 10-min intervals during rewarming period. The values are average measurements from six different experiments. Error bars show the standard errors of the mean. The cooling process lasted for 60 min.

Fig. 4 Skin perfusion as a function of skin temperature during cooling (stars) and passive rewarming (dots). The arrows show the process direction. Each two subsequent data points are separated by 1 min. (a) Hysteresis plot from a single experiment. (b) The hysteresis plot based on the average measurements from six experiments.

significantly greater than zero (26.0 ± 4.5 (SE), $P < 0.01$). The slopes of the regression lines from Eq. (1) fit of CVC to skin temperature are presented in Table 3. The interaction terms (between the flag variable and the linear and the quadratic temperature terms) were both significant, implying a real difference between the two regressions lines. The likelihood ratio test showed that the current model was significantly better than if any or all random effect terms were omitted in Eq. (1). The Q–Q plot of residuals of the regression fit meets the requirement for normality. Sample size calculations showed that the power of this study was 0.84 for a sample size of 6.

Figure 5 shows the result of the COMSOL simulation for the transient cooling temperature histories at incremental depths into the tissue.

Table 3 MMR results

	Parameter	Estimate (SE)	Significance	Confidence interval	
Cooling	Intercept	64.1 (38.3)	0.144	−29.0	157.1
	Temperature	−4.8 (3.1)	0.176	−12.4	2.8
	Temperature ²	0.2 (0.1)	0.061	−0.01	0.3
Rewarming	Intercept	−216.8 (38.2)	0.001	−309.7	−123.8
	Temperature	19.3 (3.1)	0.001	11.7	26.9
	Temperature ²	−0.3 (0.1)	0.005	−0.4	−0.1

Note: The coefficients for MMR for the quadratic function fit to the CVC versus temperature data for cooling and rewarming periods.

Fig. 5 Simulation of transient temperature at incremental tissue depths during cooling and rewarming. The dark solid graph depicts the applied surface skin temperature (T_s). The initial spatial temperature gradient was calculated for steady-state conditions prior to the start of cooling.

The simulation results exhibit a 13 °C drop in temperature following 60 min of surface cooling at the depth of 2 mm from the skin surface while the surface temperature changes by 16 °C. The simulation also displays a 20-s delay in temperature change at the depth of 2 mm.

Discussion

The goal of this study was to investigate the effect of localized cooling with a commercially available CTU on the skin temperature and CVC at a treatment site. The data show that localized cooling induces a pronounced reduction in CVC that is sustained long into a subsequent passive rewarming phase, providing there is no external means to stimulate the blood flow. Furthermore, these findings demonstrate that sustained reductions in CVC following cooling persist for an extended period of time despite the recovery of skin temperature, creating a hysteresis effect between CVC and skin temperature during successive periods of active cooling and passive rewarming. These results extend our prior research that demonstrated with many different CTUs a persistence of vasoconstriction during the passive rewarming period following localized cooling [22]. The control experiments differentiate the effect of cold exposure from the possible contributions from the instrumentation and the water flow through the cooling pad.

Localized cooling is well known to cause significant vasoconstriction and thus a reduction in tissue perfusion, although there is considerable variation in the magnitude of reduction reported by different studies [39–46]. For example, Knoblock et al. measured a reduction in perfusion of 86% and 70% at a depth of 2 mm after 7 [45] and 30 [44] min of cooling, whereas Ho et al. showed only a 26% reduction after 20 min [40]. This discrepancy may be explained in part by differences in measurement methods, mode and degree of cooling, or the depth at which perfusion was assessed.

There are only a few studies that measured the history of tissue perfusion after termination of active cooling. Yanagisawa et al. measured oxyhemoglobin as an indicator of the level of blood

circulation and showed that perfusion remains significantly depressed during the first 78–90 min following cooling [46], a result consistent with the current study. In contrast, Curl et al. showed no long-term effect of cold application on tissue perfusion after cessation of cryotherapy in rat muscle after induction of closed soft tissue injury [41]. The different experimental model and the tissue site evaluated and the influence of its physical dimensions on heat diffusion may contribute to the dissimilar results.

The mechanism for the persistence of vasoconstriction beyond the termination of active cooling is yet to be identified. Further work is needed in this area to understand the process for delayed recovery of tissue perfusion subsequent to active cooling. A candidate mechanism is via the release of a humoral agent during cooling that prevents vasodilation as skin temperature increases. Another candidate may be a direct biophysical effect of cold that endures beyond the active cooling period.

Localized cooling experiments in the forearm have been shown to cause vasoconstriction by activation of the Rho–Rho kinase pathway [18] and inhibition of the nitric oxide system [14]. Additionally, it has been shown that intact local sensory nerve and adrenergic function are required for the early and a significant portion of late stages of vasoconstrictive response to localized cooling, respectively [47,48]. These experiments are typically performed in a smaller surface area of skin, at higher skin temperatures (24 °C) and at different anatomical locations than was utilized in the current study and that are common to cryotherapy. Thus, whether similar mechanisms contribute to the results of the current study remain unknown. Furthermore, these investigations typically do not address the vasoactive response beyond the active cooling period.

The hysteresis effect between skin temperature and blood perfusion during serial cooling and warming has rarely been studied. Prior studies in our laboratory on the effect of burn injury followed by post-burn cooling on local tissue blood perfusion documented a hysteresis effect, but by a greatly differing physiological mechanism associated with protein denaturation [49]. Vuksanović et al. showed a similar hysteresis between forearm skin temperature and blood perfusion during cooling and rewarming over the temperature range of 29–38 °C with a cooling rate of 1 °C/min [50]. The current study measured the hysteresis phenomenon at lower temperatures in the knee region with an average cooling rate of 0.25 °C/min. Thus, the temperature/perfusion hysteresis has been identified for cooling and heating cycles that cover temperatures both above and below baseline. Other experiments in the authors' laboratory confirm this effect.

The hysteresis effect has been evaluated by applying a quadratic function in MMR to the data to accommodate for its nonlinear behavior during cooling and warming that is in Fig. 4. In a similar but much simpler approach, Kozelek et al. used a linear regression model to characterize hysteresis in a different aspect of cardiovascular system function [51]. The existence of hysteresis indicates that the system behaves as having a memory so that the present tissue state is not only dependent on its current condition but also its history. Thus, there is not a one-to-one relationship between tissue temperature and perfusion. Stated alternatively, the magnitude of blood perfusion is not determined solely by the present value of the temperature. A preliminary study (data not presented here) was undertaken with slower cooling and rewarming rates of 0.1 °C/min to determine whether the hysteresis depends on how rapidly temperature is changing. The data showed the hysteresis to remain even at this reduced rate of temperature change, indicating the phenomenon is at least partially independent of the process pathway. Further investigation is proceeding to more fully characterize the blood flow hysteresis that occurs during cryotherapy.

Figure 4(b) shows the general behavior of CVC with respect to temperature. For earlier times following the start of cooling, the rate of reduction of CVC is much slower compared to later times. The same pattern holds true for the slope of CVC/temperature

during the passive rewarming period. Piecewise regression was used to find the threshold temperature at which a significant change in slope takes place. The threshold temperatures were shown to be significantly different during the cooling and passive rewarming periods.

A potentially relevant issue in the experimental procedure is that the temperature and perfusion measurements are performed at different depths into the skin. Temperature is monitored at the skin surface and perfusion is interrogated over a tissue volume extending to 2 mm, but averaged at ~ 0.5 mm [21]. One might argue that the hysteresis effect between skin temperature and deeper perfusion is due to the time delay required for penetration of the cold/heat wave into the tissue. The time constant for thermal response during the rewarming period occurs in minutes, whereas perfusion remains depressed for more than an hour during the rewarming process. The simulation result presented in Fig. 5 shows the temperature histories at different depths during an exemplar cryotherapy experiment, indicating that temperature dropped by 10, 4.5, 4, 3.5 °C at depths of 5, 15, 17.5 and 20 mm into the tissue while the surface temperature reduced by 16 °C. Additionally, the simulation shows that the pattern of change in temperature at more superficial layers of tissue where the perfusion measurements was interrogated closely follows the change in temperature on the surface of the skin. Various reports on intratissue temperature via direct measurement or simulation measurements in literature support this general pattern of change in temperature during cooling and passive rewarming [52–56]. Thus, the differential in monitoring depths of temperature and perfusion within the tissue does not appear to contribute to the hysteresis effect.

There are many general examples of hysteresis between coupled properties other than temperature and blood perfusion in biology and physiology [51,57,58], including temperature-driven phenomena [59,60]. The consistency with which hysteresis between local temperature and blood perfusion has been observed in the current cryotherapy trials offers a compelling argument for its existence during cyclic cooling and heating. Given the clinical implications of creating a state of prolonged vasoconstriction, it would appear to be a feature of cryotherapy of great importance and well worthy of more extensive study.

Clinical Significance. Localized cooling is commonly used in treatment of soft tissue injury. There are numerous reports of injury associated with localized therapeutic cooling, but nearly always absent the information necessary to identify the mechanism of the injury process. The current literature lacks the time course information regarding tissue response to localized cooling, and especially after active cooling is terminated. The rationale for the current study was to contribute to a more complete understanding of tissue response to localized cooling that could lead to improvements in the practice of cryotherapy to reduce the risk of causing collateral ischemic derived injuries.

Methodological Considerations/Limitations. The current study takes a more comprehensive approach in understanding the response of tissue to localized cooling than typically appears in the cryotherapy literature and covers both active cooling and passive rewarming phases of the cryotherapy cycle. At this time, the mechanism that governs the hysteresis between tissue temperature and blood flow is not understood explicitly, but contributing factors could be vasoactive agents that remain active long after the active cooling phase is terminated. Alternate factors could be associated with direct biophysical effects of cooling. Further research is being pursued by the authors to determine the molecular mechanism(s) responsible for the observed effects.

Conclusion

The skin temperature and CVC responses to localized cooling were measured. Data showed a significant decrease in CVC during

cooling that persisted well into the rewarming period. Furthermore, a hysteresis effect between CVC and skin temperature was observed during the tissue cooling and rewarming cycle. The persistence of a significant reduction in CVC may be a contributing factor in the development of ischemic injury processes associated with localized cooling. Further investigation is needed to understand the mechanism for the persistence of vasoconstriction during rewarming and the hysteresis between CVC and skin temperature.

Acknowledgment

This research was sponsored by National Science Foundation Grant Nos. CBET 0828131, CBET 096998, and CBET 1250659, National Institutes of Health Grant No. R01 EB015522, and the Robert and Prudie Leibrock Professorship in Engineering at the University of Texas at Austin. We would also like to thank Dr. Michael Mahometa of the Division of Statistics and Scientific Computation, College of Natural Sciences at the University of Texas at Austin for his help and guidance in statistical analysis of data. Patent applications that cover certain aspects of the technologies discussed herein have been submitted by The University of Texas to the United States Patent and Trademark Office naming Dr. Khoshnevis, Dr. Brothers and Dr. Diller as inventors. Ownership rights to these patents reside with The University of Texas System. Dr. Diller has served as an expert witness for both plaintiff and defendant counsel since 2000 in numerous legal cases regarding the safety and design of existing cryotherapy devices.

References

- [1] Knight, K. L., 1995, *Cryotherapy in Sport Injury Management*, Human Kinetics, Champaign, IL.
- [2] Swenson, C., Swärd, L., and Karlsson, J., 1996, "Cryotherapy in Sports Medicine," *Scand. J. Med. Sci. Sports*, **6**(4), pp. 193–200.
- [3] MacAuley, D., 2001, "Do Textbooks Agree on Their Advice on Ice?" *Clin. J. Sport Med.*, **11**(2), pp. 67–72.
- [4] Francis, T. J., and Golden, F. S., 1985, "Non-Freezing Cold Injury: The Pathogenesis," *J. R. Nav. Med. Serv.*, **71**(1), pp. 3–8.
- [5] Francis, T. J., 1984, "Non Freezing Cold Injury: A Historical Review," *J. R. Nav. Med. Serv.*, **70**(3), pp. 134–139.
- [6] Thomas, J. R., and Oakley, E. H. N., 2002, "Nonfreezing Cold Injury," *Textbooks of Military Medicine: Medical Aspects of Harsh Environments*, Office of the Surgeon General, U. S. Army, Falls Church, VA.
- [7] Jia, J., and Pollock, M., 1999, "Cold Nerve Injury is Enhanced by Intermittent Cooling," *Muscle Nerve*, **22**(12), pp. 1644–1652.
- [8] Selfe, J., Hardaker, N., Whitaker, J., and Hayes, C., 2007, "Thermal Imaging of an Ice Burn Over the Patella Following Clinically Relevant Cryotherapy Application During a Clinical Research Study," *Phys. Ther. Sport*, **8**(3), pp. 153–158.
- [9] Brown, W. C., and Hahn, D. B., 2009, "Frostbite of the Feet After Cryotherapy: A Report of Two Cases," *J. Foot Ankle Surg.*, **48**(5), pp. 577–580.
- [10] Lee, C. K., Pardun, J., Buntic, R., Kiehn, M., Brooks, D., and Buncke, H. J., 2007, "Severe Frostbite of the Knees After Cryotherapy," *Orthopedics*, **30**(1), pp. 63–64.
- [11] Babwah, T., 2011, "Common Peroneal Neuropathy Related to Cryotherapy and Compression in a Footballer," *Res. Sports Med.*, **19**(1), pp. 66–71.
- [12] Bassett, F. H., Kirkpatrick, J. S., Engelhardt, D. L., and Malone, T. R., 1992, "Cryotherapy-Induced Nerve Injury," *Am. J. Sports Med.*, **20**(5), pp. 516–518.
- [13] Moeller, J. L., Monroe, J., and McKeag, D. B., 1997, "Cryotherapy-Induced Common Peroneal Nerve Palsy," *Clin. J. Sport Med.*, **7**(3), pp. 212–216.
- [14] Hodges, G. J., Zhao, K., Kosiba, W. A., and Johnson, J. M., 2006, "The Involvement of Nitric Oxide in the Cutaneous Vasoconstrictor Response to Local Cooling in Humans," *J. Physiol.*, **574**(3), pp. 849–857.
- [15] Johnson, J. M., and Kellogg, D. L., 2010, "Local Thermal Control of the Human Cutaneous Circulation," *J. Appl. Physiol.*, **109**(4), pp. 1229–1238.
- [16] Johnson, J. M., 2006, "Mechanisms of Vasoconstriction With Direct Skin Cooling in Humans," *AJP Heart Circ. Physiol.*, **292**(4), pp. H1690–H1691.
- [17] Minson, C. T., 2010, "Thermal Provocation to Evaluate Microvascular Reactivity in Human Skin," *J. Appl. Physiol.*, **109**(4), pp. 1239–1246.
- [18] Thompson-Torgerson, C. S., Holowatz, L. A., Flavahan, N. A., and Kenney, W. L., 2006, "Cold-Induced Cutaneous Vasoconstriction is Mediated by Rho Kinase In Vivo in Human Skin," *AJP Heart Circ. Physiol.*, **292**(4), pp. H1700–H1705.
- [19] Brown, F. T., 2006, *Engineering System Dynamics: A Unified Graph-Centered Approach*, CRC Press, Boca Raton, FL.
- [20] Noori, H. R., 2014, *Hysteresis Phenomena in Biology*, Springer, Heidelberg, Germany.
- [21] Clough, G., Chipperfield, A., Byrne, C., de Mul, F., and Gush, R., 2009, "Evaluation of a New High Power, Wide Separation Laser Doppler Probe:

- Potential Measurement of Deeper Tissue Blood Flow," *Microvasc. Res.*, **78**(2), pp. 155–161.
- [22] Khoshnevis, S., Craik, N. K., and Diller, K. R., 2015, "Cold-Induced Vasoconstriction May Persist Long After Cooling Ends: An Evaluation of Multiple Cryotherapy Units," *Knee Surg. Sports Traumatol. Arthrosc.*, **23**(9), pp. 2475–2483.
- [23] Khoshnevis, S., Nordhauser, J., Craik, N., and Diller, K. R., 2014, "Quantitative Evaluation of the Thermal Heterogeneity on the Surface of Cryotherapy Cooling Pads," *ASME J. Biomech. Eng.*, **136**(7), p. 074503.
- [24] Ramanathan, N. L., 1964, "A New Weighting System for Mean Surface Temperature of the Human Body," *J. Appl. Physiol.*, **19**(3), pp. 531–533.
- [25] Bissonnette, B., Sessler, D. I., and LaFlamme, P., 1989, "Intraoperative Temperature Monitoring Sites in Infants and Children and the Effect of Inspired Gas Warming on Esophageal Temperature," *Anesth. Analg.*, **69**(2), pp. 192–196.
- [26] Brinnet, H., and Cabanac, M., 1989, "Tympanic Temperature is a Core Temperature in Humans," *J. Therm. Biol.*, **14**(1), pp. 47–53.
- [27] Gagnon, D., Lemire, B. B., Jay, O., and Kenny, G. P., 2010, "Aural Canal, Esophageal, and Rectal Temperatures During Exertional Heat Stress and the Subsequent Recovery Period," *J. Athl. Train.*, **45**(2), pp. 157–163.
- [28] NIST, 2014, *NIST/SEMATECH e-Handbook of Statistical Methods*, National Institute of Standards and Technology (NIST), Gaithersburg, MD.
- [29] Pokala, N., 2012, "Dunnett Test for Multiple Comparisons," MATLAB Central File Exchange, accessed 26 Oct., 2013, <http://www.mathworks.com/matlabcentral/fileexchange/38157-dunnett-m>
- [30] Faul, F., Erdfelder, E., Lang, A.-G., and Buchner, A., 2007, "G* Power 3: A Flexible Statistical Power Analysis Program for the Social, Behavioral, and Biomedical Sciences," *Behav. Res. Methods*, **39**(2), pp. 175–191.
- [31] Faul, F., Erdfelder, E., Buchner, A., and Lang, A.-G., 2009, "Statistical Power Analyses Using G* Power 3.1: Tests for Correlation and Regression Analyses," *Behav. Res. Methods*, **41**(4), pp. 1149–1160.
- [32] Holmes, K. R., 1998, "Thermal Conductivity of Selected Tissues," *Biotransport: Heat and Mass Transfer in Living Systems*, K. R. Diller, ed., New York Academy of Sciences, New York.
- [33] Fiala, D., Lomas, K. J., and Stohrer, M., 1999, "A Computer Model of Human Thermoregulation for a Wide Range of Environmental Conditions: The Passive System," *J. Appl. Physiol.*, **87**(5), pp. 1957–1972.
- [34] Roselli, R. J., and Diller, K. R., 2011, *Biotransport: Principles and Applications*, Springer, New York.
- [35] Pennes, H. H., 1948, "Analysis of Tissue and Arterial Blood Temperatures in the Resting Human Forearm," *J. Appl. Physiol.*, **1**(2), pp. 93–122.
- [36] Hegarty, T. W., 1973, "Temperature Coefficient (Q₁₀), Seed Germination and Other Biological Processes," *Nature*, **243**(5405), pp. 305–306.
- [37] Dennis, B. H., Eberhart, R. C., Dulikravich, G. S., and Radons, S. W., 2003, "Finite-Element Simulation of Cooling of Realistic 3-D Human Head and Neck," *ASME J. Biomech. Eng.*, **125**(6), pp. 832–840.
- [38] Razali, N. M., and Wah, Y. B., 2011, "Power Comparisons of Shapiro-Wilk, Kolmogorov-Smirnov, Lilliefors and Anderson-Darling Tests," *J. Stat. Model. Anal.*, **2**(1), pp. 21–33.
- [39] Taber, C., Contryman, K., Fahrenbruch, J., LaCount, K., and Cornwall, M. W., 1992, "Measurement of Reactive Vasodilation During Cold Gel Pack Application to Nontraumatized Ankles," *Phys. Ther.*, **72**(4), pp. 294–299.
- [40] Ho, S. S. W., Illgen, R. L., Meyer, R. W., Torok, P. J., Cooper, M. D., and Reider, B., 1995, "Comparison of Various Icing Times in Decreasing Bone Metabolism and Blood Flow in the Knee," *Am. J. Sports Med.*, **23**(1), pp. 74–76.
- [41] Curl, W. W., Smith, B. P., Marr, A., Rosencrance, E., Holden, M., and Smith, T. L., 1997, "The Effect of Contusion and Cryotherapy on Skeletal Muscle Microcirculation," *J. Sports Med. Phys. Fitness*, **37**(4), pp. 279–286.
- [42] Sendowski, I., Savourey, G., Besnard, Y., and Bittel, J., 1997, "Cold Induced Vaso-dilatation and Cardiovascular Responses in Humans During Cold Water Immersion of Various Upper Limb Areas," *Eur. J. Appl. Physiol.*, **75**(6), pp. 471–477.
- [43] Karunakara, R. G., Lephart, S. M., and Pincivero, D. M., 1999, "Changes in Forearm Blood Flow During Single and Intermittent Cold Application," *J. Orthop. Sports Phys. Ther.*, **29**(3), pp. 177–180.
- [44] Knobloch, K., Kraemer, R., Lichtenberg, A., Jagodzinski, M., Gosling, T., Richter, M., and Krettek, C., 2006, "Microcirculation of the Ankle After Cryo/Cuff Application in Healthy Volunteers," *Int. J. Sports Med.*, **27**(3), pp. 250–255.
- [45] Knobloch, K., Grasmann, R., Spies, M., and Vogt, P. M., 2008, "Midportion Achilles Tendon Microcirculation After Intermittent Combined Cryotherapy and Compression Compared With Cryotherapy Alone: A Randomized Trial," *Am. J. Sports Med.*, **36**(11), pp. 2128–2138.
- [46] Yanagisawa, O., Homma, T., Okuwaki, T., Shimao, D., and Takahashi, H., 2007, "Effects of Cooling on Human Skin and Skeletal Muscle," *Eur. J. Appl. Physiol.*, **100**(6), pp. 737–745.
- [47] Hodges, G. J., Traeger, J. A., 3rd, Tang, T., Kosiba, W. A., Zhao, K., and Johnson, J. M., 2007, "Role of Sensory Nerves in the Cutaneous Vasoconstrictor Response to Local Cooling in Humans," *Am. J. Physiol. Heart Circ. Physiol.*, **293**(1), pp. H784–H789.
- [48] Johnson, J. M., Yen, T. C., Zhao, K., and Kosiba, W. A., 2005, "Sympathetic, Sensory, and Nonneuronal Contributions to the Cutaneous Vasoconstrictor Response to Local Cooling," *Am. J. Physiol. Heart Circ. Physiol.*, **288**(4), pp. H1573–H1579.
- [49] Ross, D. C., and Diller, K. R., 1978, "The Therapeutic Effects of Postburn Cooling," *ASME J. Biomech. Eng.*, **100**(3), pp. 149–152.
- [50] Vuksanović, V., Sheppard, L. W., and Stefanovska, A., 2008, "Nonlinear Relationship Between Level of Blood Flow and Skin Temperature for Different Dynamics of Temperature Change," *Biophys. J.*, **94**(10), pp. L78–L80.
- [51] Kozelek, P., Holcik, J., and Sedlinska, M., 2007, "Statistical Analysis of QT/RR Hysteresis in Healthy Horses," 29th Annual International Conference of the IEEE Engineering in Medicine and Biology Society (EMBS 2007), Lyon, France, Aug. 22–26, pp. 5319–5322.
- [52] Merrick, M. A., Knight, K. L., Ingersoll, C. D., and Potteiger, J. A., 1993, "The Effects of Ice and Compression Wraps on Intramuscular Temperatures at Various Depths," *J. Athl. Train.*, **28**(3), pp. 236–245.
- [53] Myrer, J., Measom, G., and Fellingham, G., 1998, "Temperature Changes in the Human Leg During and After Two Methods of Cryotherapy," *J. Athl. Train.*, **33**(1), pp. 25–29.
- [54] Warren, T. A., 2004, "Intra-Articular Knee Temperature Changes: Ice Versus Cryotherapy Device," *Am. J. Sports Med.*, **32**(2), pp. 441–445.
- [55] Holwerda, S. W., Trowbridge, C. A., Womochel, K. S., and Keller, D. M., 2013, "Effects of Cold Modality Application With Static and Intermittent Pneumatic Compression on Tissue Temperature and Systemic Cardiovascular Responses," *Sports Health*, **5**(1), pp. 27–33.
- [56] Diller, K. R., 2015, "Heat Transfer in Health and Healing," *ASME J. Heat Transfer*, **137**(10), p. 103001.
- [57] Goudkamp, J. E., Seebacher, F., Ahern, M., and Franklin, C. E., 2004, "Physiological Thermoregulation in a Crustacean? Heart Rate Hysteresis in the Freshwater Crayfish *Cherax Destructor*," *Comp. Biochem. Physiol. A*, **138**(3), pp. 399–403.
- [58] Zachariassen, K. E., 1985, "Physiology of Cold Tolerance in Insects," *Physiol. Rev.*, **65**(4), pp. 799–832.
- [59] George, N. T., Irving, T. C., Williams, C. D., and Daniel, T. L., 2013, "The Cross-Bridge Spring: Can Cool Muscles Store Elastic Energy?" *Science*, **340**(6137), pp. 1217–1220.
- [60] Inoue, I., Kobatake, Y., and Tasaki, I., 1973, "Excitability, Instability and Phase Transitions in Squid Axon Membrane Under Internal Perfusion With Dilute Salt Solutions," *Biochim. Biophys. Acta*, **307**(3), pp. 471–477.