NITRIC OXIDE SIGNALLING PATHWAYS FOLLOWING CHRONIC PASSIVE HEAT THERAPY

by

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A THESIS

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CVD is associated with vascular dysfunction and is often mediated by impairments in nitric oxide (NO), a powerful vasodilator, and increased oxidative stress. Heat shock proteins (HSP), upregulate proteins linked to improving vascular functioning such as endothelial nitric oxide synthase (eNOS) and super oxide dismutase (SOD; an antioxidant). Therefore chronic passive heat therapy (CHT) may help improve cutaneous circulation in the microvasculature. PURPOSE: To investigate the *in vivo* effects of CHT on cutaneous NO-dependent dilation and to additionally elucidate the mechanisms behind the improvement through *in vitro* cell cultures. METHODS: 18 subjects were immersed in 40°C water for 8wks, 4-5x/week to maintain rectal temperature ≥38.5°C for 60min. Prior to and following the last bout, two intradermal microdialysis fibers were inserted into the forearm and infused with lactated Ringer's solution (control) and L-NNA, to inhibit eNOS. Local skin heaters were placed at each site over the fiber and heated to 39°C at a rate of 0.1C/sec, increasing skin blood flow, which was measured using laser-Doppler flowmetry. Data are presented as the change in NO-dependent dilation from 0 to 8 weeks. Additionally post-heat therapy serum was collected at least 36h after the last session. Purchased human umbilical vein endothelial cells were cultured and exposed for 24h to either 37°C (control), direct heat at 39°C, or human sera collected at 0 and 8wks. eNOS, SOD, HSP70, and HSP90 protein expression was determined using Western blot normalized to vinculin loading control. RESULTS: Subjects' NO-dependent dilation increased significantly ($p=.0062$). There were no improvements in sham subjects. Direct heating increased SOD expression in endothelial cells by 1.23±0.10 fold ($p=0.045$), HSP70 expression by 1.32±0.03 fold ($p<0.008$), and HSP90 expression by 1.84±0.38 fold ($p=0.04$), but had no effect on eNOS (1.04±0.04 fold change, $p=0.38$). Serum exposure increased both eNOS (1.14±0.37 fold change, $p<0.002$) and SOD expression (1.29±0.09 fold change, $p<0.01$), with no change in HSP70 (0.98±0.13 fold change, $p=0.85$) or HSP90 (0.94±0.08 fold change, $p=0.51$). Thus, CHT should be considered an alternative means for improving cardiovascular health.

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Table of Contents

Bibliography 58

List of Figures

Introduction

The United States currently faces the massive threat of Cardiovascular Disease (CVD) accounting for over 30% of deaths nationally (7). This highly prevalent disease is typically characterized by vascular dysfunction. Vascular dysfunction is often caused by two major impairments in the vascular endothelium, defined as the inner lining of blood vessels. The first is a deficiency in nitric oxide (NO) bioavailability. NO is important for regulating the dilation or constriction in the blood vessels, the formation of new vessels, and preventing atherosclerosis. Therefore impaired measures of NO are highly correlated with cardiovascular risk and mortality (11). The second impairment is increased oxidative stress through the presence of free radicals. Free radicals have the ability to strip electrons from other molecules in an effort to become more stable. However the affected molecules are then made unstable and in turn will strip electrons from other molecules around them, thus resulting in a chain reaction that can cause catastrophic damage. One such affected molecule is NO, which can be bonded to the free radical superoxide to further exacerbate the vascular damage as $ONOO^-$.

However, there are means to combat these impairments. The physiological community has well established that exercise is one of the greatest preventative measures against CVD. Exercise helps to combat CVD by activating multiple signaling pathways that induce positive changes in both the vasculature and microvasculature.

One of the primary reasons exercise is beneficial to cardiovascular health is because the energy that activates the working muscles is released as heat. The resulting rise in core temperature is then known to up-regulate heat shock proteins. These heat shock proteins in turn have the ability to stabilize or upregulate many other proteins,

including those associated with improved NO bioavailability (13). Additionally it is known that exercise helps to reduce oxidative stress by restoring the balance between free radicals and antioxidants by upregulating proteins associated with improved antioxidant production. Antioxidants then reduce oxidative stress by donating an electron to the free radicals before they can continue to cause major damage to cellular membranes (2, 14). Superoxide dismutase (SOD) is one such antioxidant that can neutralize the negative effects of superoxide by binding to it. This ultimately allows for more NO bioavailability because fewer amounts of NO are being converted by free radicals into $ONOO^-$. Therefore, both of these positive implications explain why exercise would be recommended for preventing chronic pathological cardiovascular conditions.

However, in this assumption lies an inherent problem in that many patient populations are unable to fully engage or benefit from rigorous activity. Such populations can include but are not limited to spinal cord injury, diabetic, obese, or elderly patients. Many are limited from engaging in rigorous activity enough to induce major cardiovascular benefits. Therefore there becomes a desperate need for researchers to find alternative means for improving cardiovascular health for these patient groups.

Animal studies have recently shown that chronic passive heat therapy (CHT) induces extensive cardiovascular benefits, many of which display a striking resemblance to those observed after chronic exercise training (5). For our purposes, repeated use of CHT is defined as the use of hot tub, sauna, or climate chamber heat to raise the internal core temperature of the subjects. However, while the chronic effects of heat therapy have been recorded in animals, (1) far less research conducted has studied

the effects of chronic bouts of heat therapy (hot tub or sauna use) in humans over a long period of time.

Therefore the purpose of this study is to determine if chronic passive heat therapy can induce positive cardiovascular benefits in human subjects over an 8-week period of time by studying the effects of CHT on cutaneous NO bioavailability in the microvasculature. Furthermore, this study serves to elucidate the mechanisms behind the observed changes in human subjects.

For this thesis we investigated how CHT impacts the molecular pathways involved with NO signaling. This was done first through *in vivo* studies using the intact cutaneous microcirculation in the forearm. *In vivo* is defined as a study that is performed in a living organism, in this case humans. Studying the changes induced on the microvasculature is a strategic method of gauging overall cardiovascular health. This method is effective because pathologies will typically surface in the microvasculature first before presenting itself in the greater circulatory system (7). By studying changes in the microvasculature we can deduce what would likewise be occurring in the major vascular system. Furthermore, impairments in the microvasculature can prove to be detrimental to the body as it primarily serves to the deliver nutrients such as oxygen as well as uptake carbon dioxide from the tissues. The microcirculation also assists with regulating blood flow and maintaining blood pressure throughout the body. Therefore damages to the microvasculature cannot only indicate future impairments in the macrocirculation, but it can also be catastrophic in itself.

Additionally an *in vitro* component has been included for this study. *In vitro* is defined as a study that is performed in a culture dish in a laboratory setting. For this

particular study, cultured human umbilical vein endothelial cells (HUVECs) were used to elucidate the mechanisms behind the observed improvement in the microvasculature. In practice, cultured endothelial cells would be exposed to simulated conditions that the human subjects' cells would experience. By simulating the conditions that subjects' endothelial cells were exposed to in a controlled laboratory setting, we would be able to reveal the signaling cellular pathways that account for the adapted NO bioavailability. Such a study is important for acutely understanding how passive heat therapy can be used to induce cardiovascular benefits.

Therefore, by combining both an *in vivo* as well as *in vitro* component for this study, we can simultaneously understand how chronic passive heat therapy can be used as a beneficial method of improving cardiovascular health, as well as the mechanisms in which it does so. The reasoning behind these methods are that cultured endothelial cells allow us to see the mechanisms behind the increased NO that cannot be otherwise observed in humans. Such understanding could potentially be used for the direction of future studies as well as how to best implement CHT as a treatment for patient populations.

Literature Review

Heat Therapy

Multiple studies by Dr. Horowitz have highlighted the improvements in cardiovascular health post heat acclimation in animal studies. In one such study Horowitz observed that heat acclimation increased the bioavailability of HSP70. Additionally they found that heat acclimation resulted in improved protective effects that could delay thermal injury by preventing cell death. Therefore, if such results can be concluded by animal studies, the next step would be to study the effects of CHT in humans and observe whether or not these results are replicable (5).

A study by Akasaki et al. also studied rats to determine the effects of heat therapy through repeated infrared dry sauna exposure. The heat therapy mice were placed in the saunas 41 for 15 minutes and then at 34 for 20 minutes daily for 5 weeks. Using Laser Doppler imaging, which will be described in the *Microdialysis Skin Study* section, they observed the effects of heat therapy on perfusion. They also found that capillary density had significantly increased in the heat therapy group. Lastly, they also studied the effects of heat therapy on endothelial nitric oxide synthase (eNOS) expression. This was done in order to see if the protein was possibly involved in the heat therapy induced angiogenesis. They concluded that the angiogenesis that the greater capillary density could in fact be attributed to eNOS which was upregulated by the heat therapy protocol. (1)

Among the very limited research conducted in humans that examined the effects of long term CHT is a study conducted by Laukkanen et al. in Finland. Researchers in

this study sought to understand the association between sauna bathing and fatal cardiovascular events (heart attacks) over an extended period of time. Around 2,600 men aged 42 to 60 were given baseline examinations between the years of 1984 and 1989 to evaluate their frequency of sauna bathing. In the years after, checking hospital documents and death certificates followed up all deaths. There were no losses to follow-up (8). They found that subjects who engaged in sauna bathing experienced a lower risk of CVD and coronary heart disease. For subjects practicing 2-3 sauna sessions per week, their risk of a fatal coronary heart disease event was 23% lower. Furthermore, subjects who practiced 4-7 sauna baths per week experienced 48% lower risk. All of the findings for this study were significant (8).

However multiple barriers must be overcome when studying CHT for the purpose of being a potential method of improving cardiovascular health in patient populations. One such barrier is the fear that hot tubs are unsafe for patient populations. Tei et al. therefore conducted a study evaluating the responses to warm-water and sauna baths in 34 subjects already experiencing chronic heart failure. Subjects were immersed in either 41°C water to the sub-clavicular level for 10 minutes or an infrared-ray sauna for 15 minutes depending on the study day. Measurements of subjects' hemodynamic changes were monitored before, during, and for 30 minutes after the study. They found that none of the subjects experienced dyspnea (labored breathing), angina pectoris (chest pain due to lack of oxygen supplied to the heart), or arrhythmia (abnormal heart rhythm) during any of the 68 trials. Alternatively they found that cardiovascular hemodynamics improve after CHT protocols in patients experiencing chronic heart

failure. This shows that when monitored, using hot tubs cannot only be a safe practice in patient populations but also incredibly beneficial (15).

Microdialysis Skin Study

Cutaneous hyperemia, or rapid local skin heating has been widely used by researchers to assess microvascular function (12). Dilation in these vessels are highly dependent on NO (NO-dependent dilation). Furthermore because NO-dependent dilation can become impaired under a variety of disease conditions, it is considered a representative of globalized microvascular functioning (4,5).

During cutaneous hyperemia, multiple factors contribute to the general local heating response. Therefore, Choi et al. sought to differentiate the CVC responses dependent upon endothelial-derived hyperpolarizing factors versus NO-dependent dilation. By comparing nine heating protocols they were able to establish that locally heating the skin to 39°C was the best approach for isolating NO-dependent dilation (4). This approach was therefore used for the purposes of this study.

Laser Doppler flowmetry is a technique that can be used to determine the cutaneous blood flow through the microvasculature. Combined with the mean arterial pressure, cutaneous vascular conductance (CVC) can be calculated in order to evaluate microvascular functioning. The microvasculature will be used for this study because of its easily accessible location. Furthermore, the microvasculature is representative of overall cardiovascular functioning (4,11).

Microdialysis fibers can be used for the infusion of drugs in order to determine the response by a local capillary bed during the local heating response. The semi-permeable membrane allows for the perfused drugs to interact with the bed and induce

various experimental conditions. The laser in the Laser-Doppler flow meter unit can then be used to measure the blood flow through that capillary bed after the drug response. Laser Dopplers work by projecting a light source into a tissue, the distribution of that light can then be used to estimate blood flow.

Local heating typically results in three characteristic responses (11). Immediately after heating, the skin's CVC will increase up to a *peak value*. This peak will not be evaluated for this study, as it is typically characteristic of an axonal reflex rather than NO dependent. The second response is a *nadir.* This portion of the response occurs when the axonal response is simultaneously receding while the NO-dependent dilation is increasing. The last part of the response is the *plateau*, which is almost entirely NO dependent.

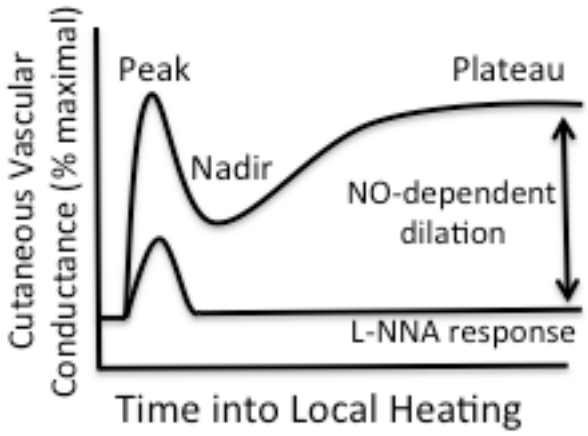


Figure 1: Typical response to local heating

L-NNA is a drug that can be used to wipe out the NO response to local heating. In pilot data it was determined that 10mM was the lowest dosage given that could induce a maximal local heating response. This is accomplished by non-specifically inhibiting eNOS, the enzyme that is responsible for NO production. By utilizing L-

NNA, any increase in CVC that would have been attributed to NO is hereby blocked. Therefore by comparing an L-NNA site to a controlled local heating plateau, all CVC between those two measures can be attributed to NO, defined as NO-dependent dilation.

Cellular Study

Cellular cultures employing human cells can be used to easily deduce what is likely occurring in the human body without the need of biopsies or other invasive procedures. This study utilized purchased human umbilical vein endothelial cells (HUVECs). The HUVECs will be grown and cultured in the lab before being exposed to the experimental conditions.

For the purposes of this study, HUVECs will be exposed to direct heating and serum from heat acclimated subjects. This is in an effort to simulate the effects of heat therapy in cultured cells to what was likely experienced *in vivo* by the endothelial cells.

In this study we will be observing four specific proteins: Endothelial nitric oxide synthase (eNOS), which breaks down L-arginine to form L-citruline and NO as a byproduct. Superoxide dismutase (SOD), an antioxidant. Heat shock protein 90 (HSP90), which stabilizes eNOS. Lastly, heat shock protein 70 (HSP70), which upregulates the production of SOD.

Western blots are a widely accepted method of evaluating protein expression after cells are exposed to experimental conditions. Mahmood et al. explains that by utilizing this technique, researchers will be able to identify specific proteins in the cells. Using gel electrophoresis, proteins are separated based on molecular weight. A band for each protein is then produced on a membrane, which can be visualized by adhering to

antibodies. The thickness/darkness of the band thus indicates the amount of protein present. (10) Western Blots for this study will be used to study the changes in protein expression of eNOS, SOD, HSP70, and HSP90 normalized to a vinculin loading control.

Methods

Subjects

For this study we recruited 76 subjects. Of those 76 subjects assessed for eligibility, 27 were randomized into either the heat therapy group (n=13) or the sham group (n=14). Of those subjects (n=8) of the heat therapy subjects or (n=10) of the sham subjects were analyzed. All subjects were young (18-30 yrs), healthy, but otherwise sedentary. Sedentary is defined as engaging in less than 2 hours of cardiovascular exercise per week. All subjects were pre-screened prior to the engagement in any protocol. This study screened for cardiovascular diseases in the subjects' family histories. Additionally all subjects on prescription medications, with the exception of oral birth control, were excluded from this study. During the pre-screen all subjects were made aware of the protocols the study required. All subjects were told that they could leave the study at any time and would be compensated for the time they had participated. Lastly, signatures of informed consent were gathered as prescribed by the Declaration of Helsinki. Institutional Review Board approval was acquired for all protocols pertaining to this study (See Appendix A).

Heat Therapy

Procedure

Prior to coming to the lab, all subjects were asked to collect a urine sample after waking and before drinking any fluids/consuming food. This standard was set to ensure that the sample would be a credible representation of the subjects overall hydration. Urine specific gravity was then measured in the lab with the acceptable level being set

at ≤1.02. All subjects falling above this standard were asked to consume 5ml/kg of their body weight. Nude body weight was obtained prior to rectal probe instrumentation. After recording body weight, subjects instrumented themselves with a rectal probe (YSI Series 400; Yellow Spring Instruments, Yellow Springs, OH) that was inserted ~10cm beyond the anal sphincter.

Subjects were then immersed in 40.5°C hot tub. All subjects were asked to sit with the water level up to their clavicles in order to drive rectal temperature up to 38.5°C. Once the target rectal temperature of 38.5°C was reached, subjects were allowed to sit up with the water level at their waist. This target temperature was chosen because it is considered the threshold for heat shock protein activation.

Subjects stayed in the hot tub for an hour after the target temperature was reached or until 90 min total of immersion, whichever came first. Subjects were then asked to sit out of the tub for 10 minutes post immersion until rectal temperature fell below 38.5°C.

Throughout the entire immersion and for the 10 minutes following, rectal temperature, heart rate, water temperature, subject position, and general notes were recorded every 5 minutes. This was done in order to ensure that the subject stayed within safe levels of internal temperature. Additionally the weight of fluids consumed during the immersion was also recorded.

At the conclusion of the 10 minutes post immersion, subjects' nude body weight was again recorded. Using the pre and post weights, as well as factoring out fluids consumed, sweat loss was calculated. If a subjects' post body weight was >1% pre body weight they were asked to consume enough water so that there was no more than 1%

body weight loss. This was to ensure that subjects were euhydrated at the conclusion of the study for their own safety.

This protocol was repeated for each subject 4-5 times/week for 8 weeks, a total of 36 hot tub sessions. Protocol was repeated in sham subjects with the exception of the tubs being filled with 36.5°C water and rectal temperature was not driven up to 38.5°C. Rather, rectal temperature was maintained at baseline levels throughout the immersion.

Microdialysis Skin Study

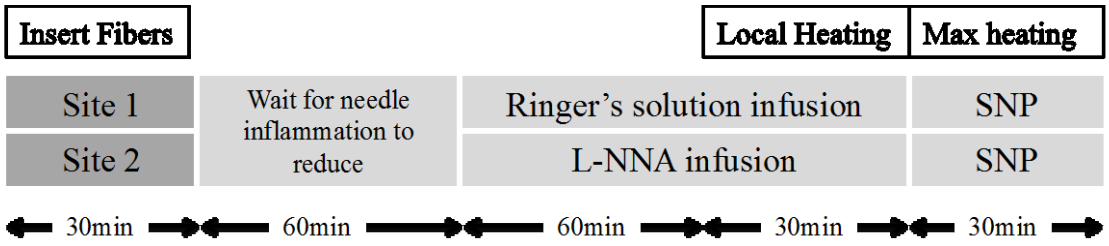


Figure 2: Skin study protocol

Procedure

Prior to the first hot tub session and 36-72 hours after the last session, a skin study was conducted. Female subjects were asked to provide a urine sample beforehand to rule out pregnancy. Additionally all subjects were screened to be sure that the subjects had no food (4hrs), caffeine or alcohol (>12hrs), medications/vitamins/supplements (24hrs), and exercise (24hrs) before the planned study day. Height and weight were also recorded.

Subjects were told to sit in a semi-recumbent chair while resting their left arm on a table. On the ventral forearm 2 skin sites for fiber placement were then located ≥4cm apart from each other. Care was taken to avoid veins, hair, or sun damaged areas as these factors could disrupt the physiology. Once the spots were located a 25-gauge

needle was embedded 1-2mm under the skin and exited ~25 mm away. A microdialysis fiber (MD2000, 30-kDa cutoff membrane; Bioanalytical Systems, Inc., West Lafeyette, IN, http://www.basinc.com/products/iv/probes_linear.php or CMA 31 Linear Probe, 55-kDa cutoff membrane; CMA Microdialysis AB, Kista, Sweden, http://www.microdialysis.com/us/products/probes/cma-31-linear), which is a permeable fiber that can infuse intended drugs, was then threaded through the needle. Once in place, the needle was removed leaving the permeable fiber in place under the skin. This procedure was repeated for the second site.

Lactated Ringers, a solution comparable to saline (salt water), was infused through the fastened fibers. Infusion proceeded at a rate of 2μL/minute. A check was done to ensure that the fibers were perfusing properly throughout the duration of the study.

Drugs were mixed while the subject rested in place for 1 hour in order for the inflammation in the forearm to recede. During this time the subject was instrumented with a blood pressure cuff on the left arm (CardioCap; Datex-Ohmeda, Louisville, CO). An initial blood pressure and heart rate was recorded in addition to one after 20 minutes of rest. Blood pressure was taken every 5 minutes.

During the last 15 minutes of rest, local heaters (SH02 Skin Heater/Temperature Monitor; Moor Instruments, Axminster, UK) and Laser-Doppler probes (MoorLab; Moor Instruments) were placed over the two fiber sites in addition to one control site. Once sure that the inflammation had passed and stable values were recorded, all local heaters increased temperature to 33°C. This temperature was chosen in order to control for the vasoconstriction that occurs due to the skin being regularly exposed to colder

conditions. Pre-drug baseline was then recorded for 5 minutes. Additionally BP and HR were recorded.

After pre-drug baseline, the Lactated Ringers syringes were replaced with Lactated Ringers at Site 1 and L-NNA at Site 2. Site 3 was left as a control. All sites were determined randomly.

After post-drug baseline, all local heaters increased temperature to 39°C at a rate of 0.1/second. Skin blood flow was recorded throughout this process until all sites hit a stable plateau for at least 5 minutes.

Lastly the local temperatures of the heaters were increased to 43.5°C at a rate of 0.1/second. Additionally, all syringes were replaced with 56mM sodium nitroprusside (SNP) and perfused through the fibers. This is a substance that can be used to lower blood pressure and induce vasodilatation. These two methods were used in combination to ensure that all sites displayed maximal blood flow. The study concluded when a plateau was reached in the observed skin blood flow.

Statistical Analysis

Baseline, initial peak, nadir, and plateau cutaneous vascular conductance in the Ringer's site were compared using two-way RM ANOVA with factors of phase during local heating and weeks into chronic heat therapy (0wks vs. 8wks). The values for CVC were calculated by dividing the observed Laser-Doppler flux values by the mean arterial pressure acquired by the blood pressure cuff. All CVC values were then normalized as a percentage of maximal CVC. Pairwise comparisons of the %CVC max were then made using Student-Newman-Keul's post-hoc test. NO-dependent dilation was compared

across weeks into heat therapy using Student's paired t-test significance was set to $P^{*}<0.05$.

Drug Mixing

Drugs were mixed during the subject's initial hour of rest. Two drugs were mixed for the local heating portion of the study. For the first 10ml syringe, labeled 10mM L-NNA, 0.0110g L-NNA was weighed out in a clean beaker. 5ml Ringers was then added in addition to a small stir bar. The solution was stirred at a high speed at low heat until completely dissolved (20min). Solution was then pulled up into the 10ml syringe labeled 10mM L-NNA.

For the max CVC portion of the study 0.0167g SNP was weighed out into a clean beaker. 1ml of Ringers then added and whirled until fully dissolved before being pulled back up into a 3ml syringe. Drugs were filtered through a 5μm filter before being transferred to fill three 1ml syringes with 56mM SNP. All syringes containing SNP were then stored in dark plastic until time of use for drug infusions.

Cellular Study

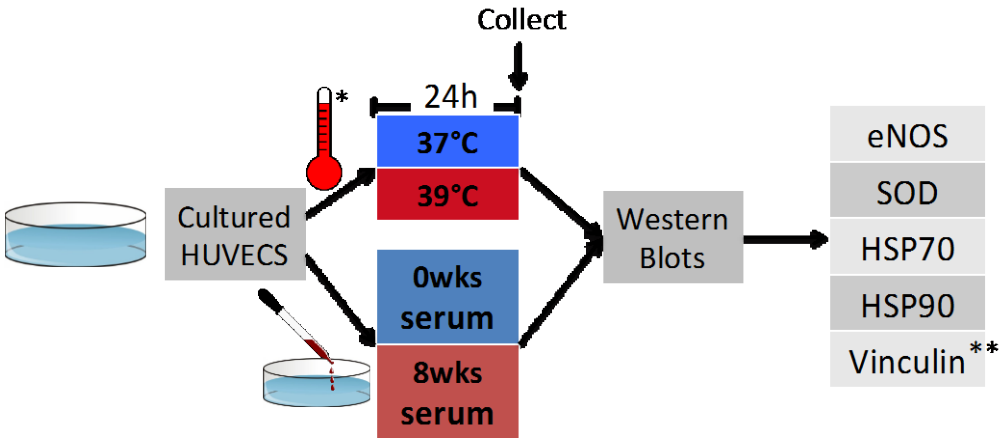


Figure 3: Cellular study protocol

Procedure

Venous blood was drawn prior to the first hot tub bout (0wks) and 24-48 hours following the last immersion (8wks). As a control, all subjects were fasted for 4 hours and had abstained from any caffeine or alcohol 12 hours prior to the draw. Additionally subjects were asked to refrain from any medications or exercise 24 hours prior to the study. Samples were drawn into serum separating vacutainers and then separated by centrifugation. All blood samples were stored using standardized preservation protocols at -80°C for later use.

Human umbilical vein endothelial cells (HUVECs) were grown and cultured using standardized procedures, and were used in experiments after 2-3 passages and following a 4h serum starve. All HUVECs were then plated and exposed to 1 of 4 conditions. For the direct heating portion of the study, cells were incubated at either 37°C (control) or 39°C (matched to the desired internal temperature set for the CHT

protocol). Cells were then kept in either of these two conditions for 24 hours and then collected for further analysis.

For the serum exposure portion of the study, cells were exposed to 10% serum (100µl in 1ml of culture medium), which had been collected at either 0wks (control) or 8wks (heat acclimated). Once again cells were incubated in these conditions for 24 hours at 37°C and then collected.

Western Blotting

Using electrophoresis on 4-20% SDS polyacrylamide separating gels (Life Technologies, Grand Island, NY), 20-50µg of protein were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA). Ponceau was used to stain the membranes before they were incubated for 1 hour in Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE). This was done in order to verify that the proteins were transferred onto the membrane successfully. The membranes were then further incubated overnight at 4°C. During this time the membranes were set in blocking buffer that contained primary antibodies (listed below). The next day the membranes were washed and incubated with their respective secondary antibodies (LI-COR Biosciences). This was conducted for 1 hour at room temperature. The bands shown on the membrane were analyzed using LI-COR Odyssey infrared imaging system (LI-COR Biosciences) in order to be quantified using LI-COR Image StudioTM software.

Primary antibodies for HUVECs were: 1) anti-endothelial NO synthase (eNOS) (1:1,000; Cell Signaling Technology, Danvers, MA) 2) anti-superoxide dismutase-2(MnSOD/SOD2) (1:1,000; Sigma-Aldrich, St. Louis, MO) 3) anti-Hsp90 [S88] (1:200;

Abcam, Cambridge, MA) 4) anti-Hsp70 [BRM-22] (1:5,000; Abcam), and 5) anti-vinculin (loading control; 1:1,000; Cell Signaling Technology).

Statistical Analysis

Results were acquired from the Western Blots relative to the vinculin loading control (a method of establishing consistencies among samples). Changes in protein expression of eNOS, SOD, HSP70 and HSP90 were then compared using a Student's paired t-test for the serum exposure and an un-paired t-test for the direct heating results. Significance was set to $P^{*}<0.05$

Results

Microdialysis Skin Study

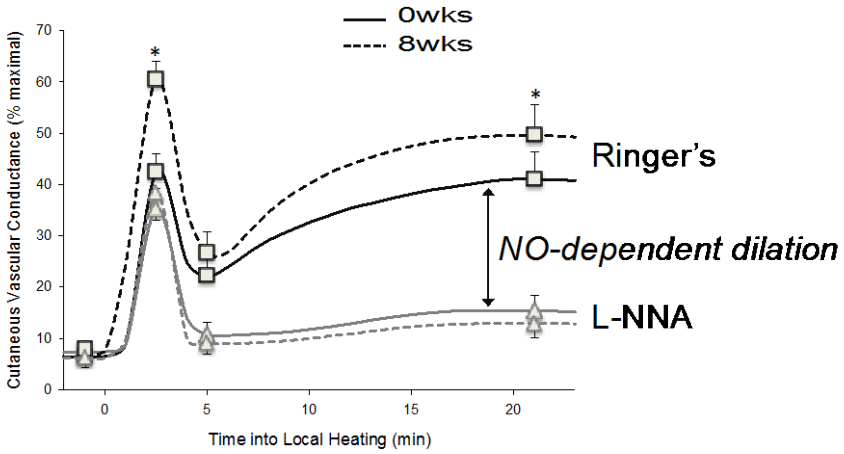


Figure 4: Effects of heat therapy on local heating response

Heat therapy significantly increased the peak value and plateau during the local heating response ($p<0.0001$ and $p=-.012$ respectively). No significant change found in sham subjects' peak ($p=.099$) and plateau ($p=0.22$).

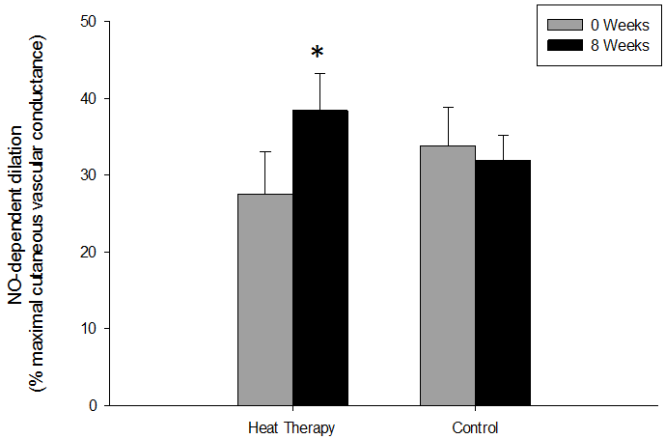


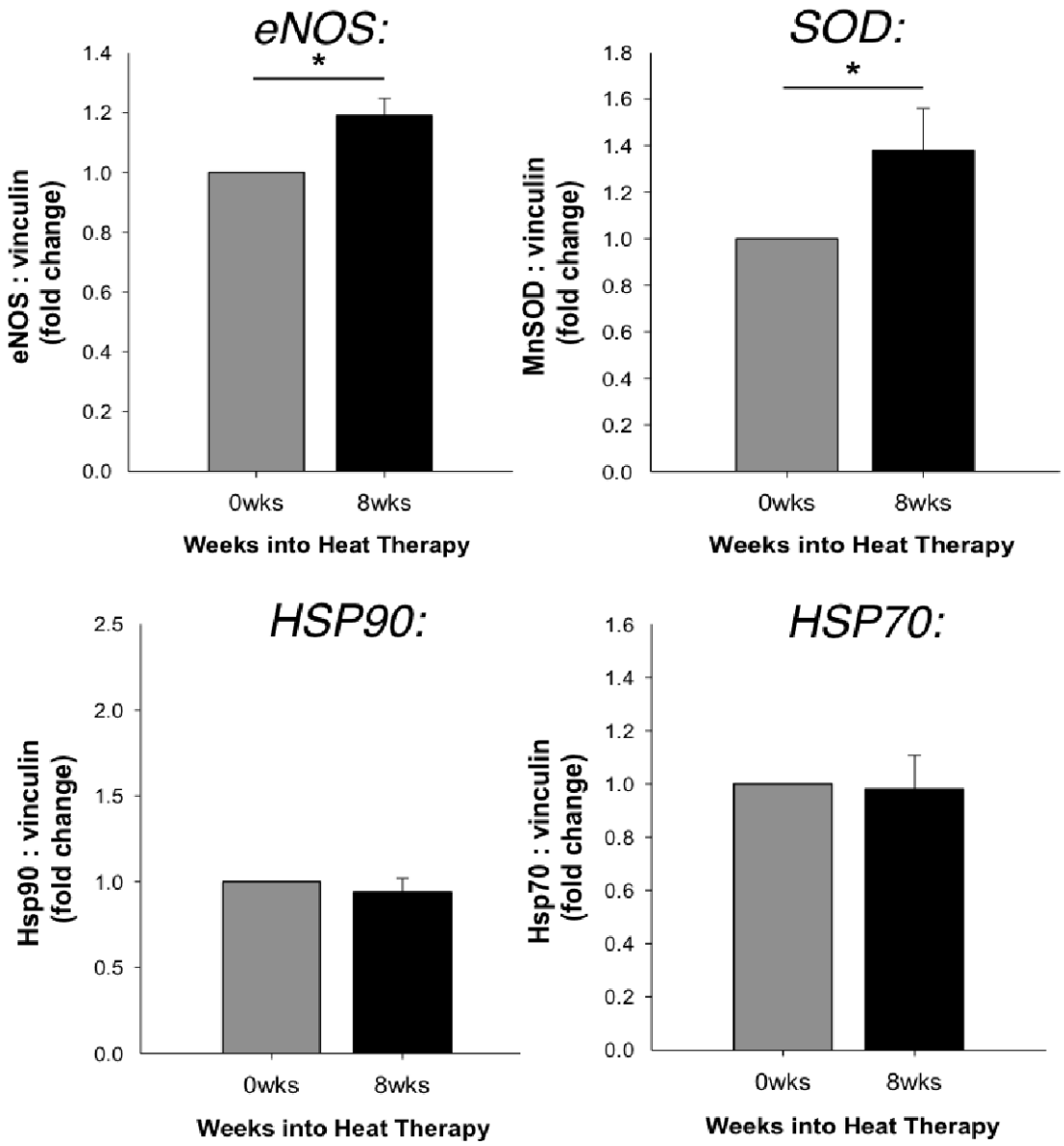
Figure 5: Effects of heath therapy and sham conditions on NO-dependent dilation

Heat therapy significantly improved mean value of NO-dependent dilation ($p=0.0062$), sham subjects experienced no significant change ($p=0.32$).

Heat therapy adjusted the local heating response by significantly increasing both the initial peak and plateau values ($p<0.0001$ and $p<0.01$ respectively). Even though the initial peak is not entirely NO dependent, there was still a significant difference between the control and the L-NNA site. This indicates that there must have been some change in NO bioavailability. The plateau site supports this conclusion because the significant change between 0 and 8 weeks indicates that there was a dramatic change in NO bioavailability in the microvasculature. This can be inferred because much of the plateau response occurs due to NO-dependent dilation. Important to note, NO-dependent dilation increased in all subjects. Conversely no significant change in local heating response was observed in in the sham subjects.

This justification is further supported by the significant increase in NO-dependent dilation ($p=0.0062$), reported as a percentage of maximal CVC. Sham subjects once again did not experience any significant change.

Cellular Study: Serum Exposure



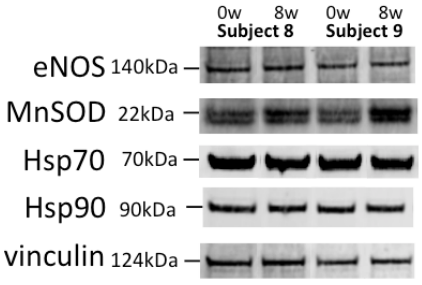


Figure 6: Effects of serum exposure on protein expression

Serum exposure significantly increased eNOS expression by 1.14±0.37 fold change, $p<0.002$, and SOD expression by 1.29±0.09 fold change, $p<0.01$. Serum exposure had no effect on HSP90 (0.94±0.08 fold change, $p=0.51$) and HSP70 (0.98±0.13 fold change, $p=0.85$).

Cellular Study: Serum Exposure Continued

After cells were exposed to 24 hours of exposure to heat acclimated serum, there was a significant increase in eNOS protein expression ($p<0.002$). Additionally we observed a significant increase in SOD expression after the exposure ($p<0.01$). Conversely neither HSP90, nor HSP70 expression were increased after the exposure to serum ($p=0.51$ and $p=0.85$ respectively). These results can be observed in the example blots from cultures using subject 8 and subject 9's zero and eight-week serum. An increased darkness or thickness of the band indicates increased protein expression. Such results indicate that eNOS and SOD can be upregulated through mechanisms independent of intracellular effects of direct heat. Therefore there must be other circulating factors upregulated by passive heat therapy that accounted for these improvements.

Cellular Study: Direct Heat Exposure

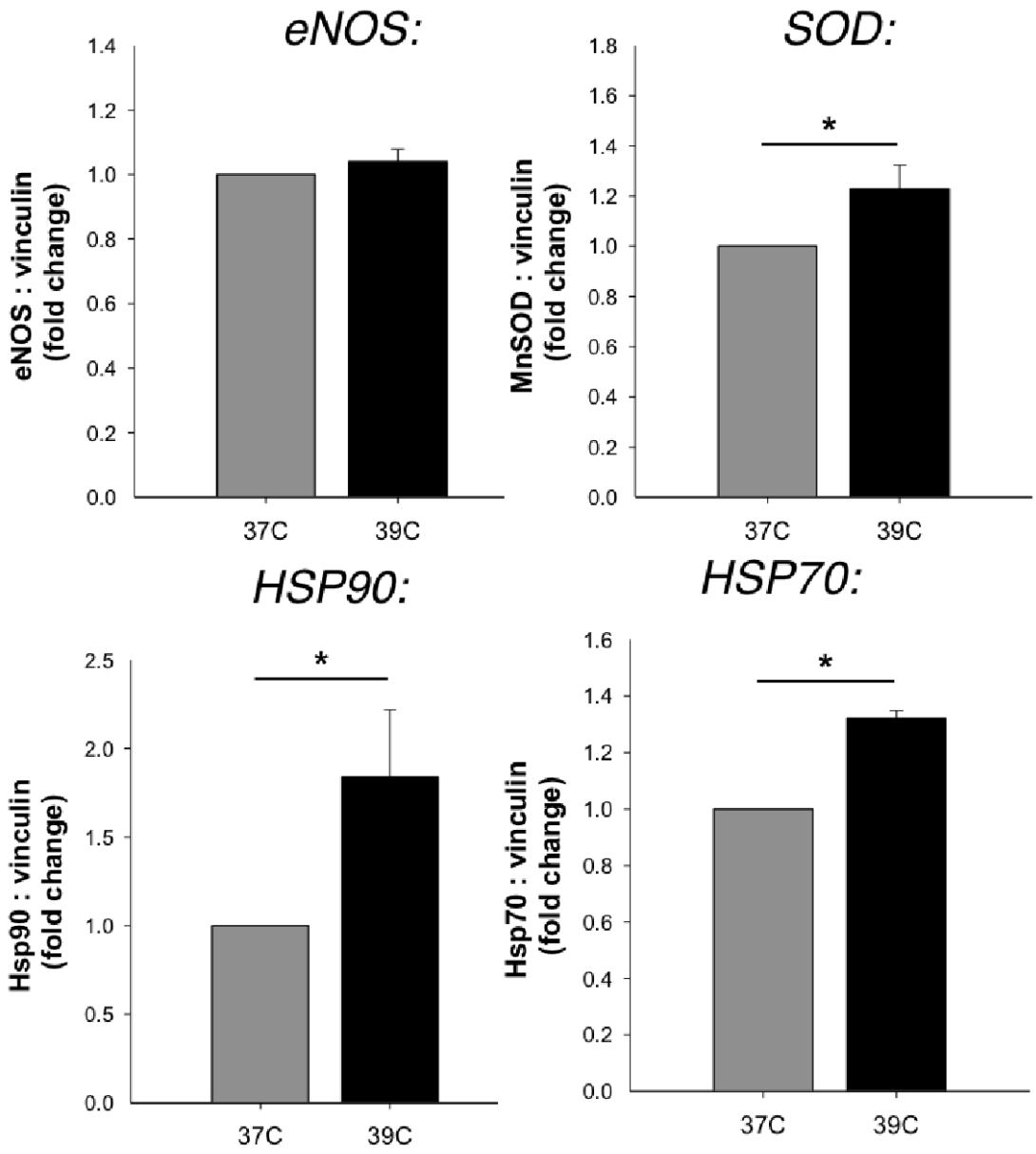
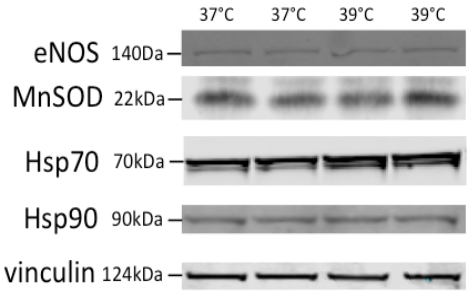


Figure 7: Effects of direct heat on protein expression



Exposure to direct heat significantly increased SOD by 1.23±0.10 fold (p=0.045), HSP90 by 1.84±0.38 fold (p=0.04), and HSP70 by1.32±0.03 fold (p<0.008) .eNOS expression did not increase. (1.04±0.04 fold change, p=0.38).

Cellular Study: Direct Heat Exposure Continued

HUVEC exposure to direct heating resulted in a significant increase in SOD expression ($p=0.045$), but no significant change was found in eNOS protein expression ($p=0.38$). Furthermore, in contrast to the serum exposure, direct heating resulted in significant increases in both HSP90 and HSP70 ($p=0.04$ and $p<0.008$ respectively). The blot examples display the contrast in band thickness for four samples that either exposed to heating at 37°C or 39°C. These results indicate that passive heat can induce an upregulation of SOD, HSP70, and HSP90 through mechanisms mediated by direct intracellular effects of heat.

Discussion

The purpose of this study was two-fold. The first component was to observe the effects of long-term exposure of bouts of heat on NO bioavailability in the microvasculature. To the best of our knowledge this is the first study to observe the mechanisms behind the effects of CHT in the microvasculature. Our primary finding was that following the 8-week passive heat protocol, the local heating response in the microvasculature significantly improved. This was largely due to the increased amounts of NO in the microvasculature, indicated by the significant increase in the plateau response at the control site with no difference in the L-NNA site.

Consistent with what was mentioned previously, a change in the difference between the L-NNA response and the Ringer's/control site's plateau is indicative of a change in NO bioavailability. As seen in Figure 4, the difference between the two responses significantly increased after the 8-week therapy. This improvement therefore likely indicates that there must be greater amounts of NO in the microvasculature that are allowing the vessels to dilate. Additionally it means that the NO present is actually functioning to dilate those vessels. It is significant to note that the improvements were found in all the heat therapy subjects.

When there is a rise in core temperature and therefore an increase in blood flow to the skin, we can suspect that such improvements can be attributed to two factors. The first is that this dissipation leads to the increase in shear stress, a mechanical force caused by increased blood flow through the vessels, which in turn is largely responsible for the acute change in diameter of the vessel. Shear stress is known to induce many benefits in the vessels, one of which is the phosphorylation and thereby activation of

eNOS (16). An upregulation of eNOS expression therefore results a greater amount of NO production in the vessels (1). The second factor is the resultant increased temperature in the skin due to the blood flow. This increase in skin temperature can lead to the activation of heat shock proteins that are associated with increased NO bioavailability.

However, we could not have known for certain the pathways of the improved bioavailability without the second component of this study. Therefore the second purpose was to elucidate the mechanisms behind the observed improvements using cellular cultures in simulated and controlled environments.

Exposure to serum from subjects after the 8-week protocol was shown to significantly improve both eNOS and SOD expression. However, both heat shock proteins 70 and 90 did not improve. Such findings indicate that there must be unknown circulating factors in the blood samples that were upregulated after heat therapy. In turn these upregulated factors induced an increased amount of eNOS and SOD, independent of heat shock protein mediated intracellular effects.

Conversely, direct heating significantly improved the expression of SOD, HSP70 and HSP90 while eNOS expression remained unchanged. This indicates that the three former proteins can all be upregulated by heat induced intracellular effects. This makes sense when considering that heat is well known to upregulate heat shock proteins (13). However it also notably shows that eNOS is not upregulated directly by heat but by other factors in the blood that have been upregulated by long-term heat exposure.

These results indicate that it is the rise in internal temperature that upregulated the expression of HSP70 and HSP90 in our heat therapy subjects. These results are

consistent with what is seen in exercising populations. It is likely the increase in HSP70 from the direct heating that upregulated the production of SOD (13,14). These improvements are important because the increased amount of antioxidants can therefore combat oxidative stress by neutralizing superoxide in the blood stream. This neutralization of free radicals allows for the NO produced to continue to function properly. Furthermore, while there may not have been an increase in expression of eNOS from direct heating, the dramatic increase in its co-factor HSP90 is likely one of the reasons that allowed for the increase in NO production. This is because more HSP90 was available to be a cofactor for eNOS, which is necessary for the production of NO.

The eNOS protein was unique from the other studied proteins in that it was not upregulated by direct heating, but rather solely from the exposure to the subjects' serum. This means that there was another factor we were unaware of in the subjects' blood that was upregulated by the heat therapy. Having increased circulation to the skin allowed for the circulating factors to upregulate the expression of eNOS. The elucidated mechanisms are therefore described in our working model in Figure 8.

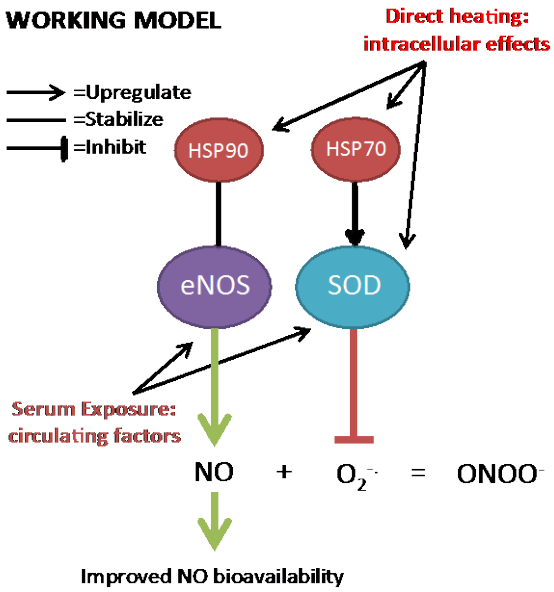


Figure 8: Working model of NO signaling pathway

Lastly, no improvement in the sham subjects was observed after the 8-weeks of thermoneutral water immersion. A sham group was used for this study rather than a time control group in order to rule out the possibility that it is the immersion in water that induced the observed results. Because only the hot water immersion resulted in improved NO-bioavailability, it can be concluded that it was the passive exposure to heat that induced the positive changes in the microvasculature.

Such findings indicate that chronic passive heat therapy is an effective way of improving cardiovascular functioning in the microvasculature. On a larger scale these results suggest that CHT can induce positive physiological responses in the greater cardiovascular system. By having upregulated amounts of HSPs, eNOS, and antioxidants such as SOD, both impaired dilation and oxidative stress, factors that can lead to vascular dysfunction, can be attenuated leading to overall improved cardiovascular health.

Limitations

There were a few limitations to this study. All of the subjects were young and healthy. Therefore further studies must be conducted before we can fully claim that heat therapy through hot tubs can improve cardiovascular health in patient populations. However, we chose to study the sedentary population because we questioned whether or not CHT could make healthy subjects even healthier. Other exercise studies have used the sedentary populations with similar goals and intentions.

Additionally we used the cutaneous microvasculature in the forearm to make claims about global microvascular health (7). However, in order to reasonably adjust for this limitation, we had the subjects always sit in the hot tub with their study arm out of the water. By doing so, we concluded that any changes in the microvasculature would have been systemic rather than from local responses to direct heating from the hot tub.

Conclusion

In conclusion, our study suggests that exposure to chronic passive heat therapy will result in greater amounts of NO bioavailability. NO will then in turn help with reducing the amount of stress on the cardiovascular system by acting as a powerful vasodilator and improving overall vascular function.

The *in vitro* component of this study elucidated the mechanisms behind the improvements in NO bioavailability. Both the effects of circulating factors and intracellular effects of heat either directly or indirectly allowed for increased production and usage of NO. These results likely underlie the observed improvements found in vascular function during the skin portion of the studies.

Future studies may benefit from investigating the effects of passive heat therapy in spinal cord injury, diabetic, or obese patient populations. Additionally on the cellular component, adding an assay to evaluate whether or not there was an increase in eNOS functionality could be beneficial for cementing the reason for the improvements in NO-dependent dilation.

Therefore it can be concluded that chronic exposure to passive heat, should be further investigated as a means of improving cardiovascular health in patient populations. Perhaps hot tub or sauna use can become a clinical alternative for lowering the risk of cardiovascular disease in populations unable to fully engage or benefit from exercise.

Appendix A: Informed Consent Documents

Group 1: Able-bodied Heat Therapy

TITLE: "Chronic heat exposure and cardiovascular health"

INVESTIGATORS: ***Vienna E Brunt, M.S., Dr. Christopher T Minson, and colleagues***

APPROVED BY INSTITUTIONAL REVIEW BOARD: ***February 5, 2014***

This is an important form. Please read it carefully. It tells you what you need to know about this study. If you agree to take part in this research study, you need to sign this form. Your signature means that you have been told about the study and what the risks are. Your signature on this form also means that you want to take part in this study.

Why is this study being done?

Cardiovascular disease is the number one cause of death in the United States. Exercise is a potent means of improving cardiovascular health, but not all patient populations are able to exercise effectively. There is high demand for novel therapies to better manage cardiovascular risk in these patients. Heat exposure can have many beneficial effects on the cardiovascular system. As such, long-term heat exposure (i.e. sitting in a hot tub 4-5x per week) may provide an alternative means to exercise for improving cardiovascular health. This project will assess the benefits of long-term heat exposure on the health of the vasculature and on the cellular pathways that improve vascular health, which is important as the majority of cardiovascular diseases affect the arteries. We will measure various biomarkers of vascular health before and after 8 weeks of heat exposure in able-bodied individuals and patients with spinal cord injury. SCI patients are a population with elevated cardiovascular risk who have limited exercise capabilities, and who may be able to utilize chronic heat exposure as an alternative to exercise training for improving cardiovascular health. Additionally, prehypertensives represent another patient population with elevated cardiovascular risk whose cardiovascular health may be affected by chronic heat exposure.

We will address the following questions in this study:

- How does 8 weeks of passive heat exposure affect cardiovascular health, as measured by various biomarkers of cardiovascular function?
- How does 8 weeks of passive heat exposure affect levels of factors circulating in the blood and located within muscle tissue that are important for cardiovascular health?
- Do effects differ between healthy normotensive able-bodied individuals and spinal cord injury patients?

What will happen in the study?

1. If you are interested in participating in the study, we will schedule an appointment with you to meet with one of the investigators of the study to discuss the project, to see the laboratory, and to read this form. If we have scheduled this appointment, it means you meet all initial subject criteria (based on initial phone and/or email conversations).
2. Additionally during this initial session, you will fill out a health history form and may meet with a physician, so that we can ensure you are healthy enough to participate in the study. In addition, the physician will be available to you to answer any medical questions or concerns you may have throughout the duration of your participation in the study. This visit should last about 60 minutes.
3. We will assign you to a subject group. If you are reading this form, you are in the **able-bodied heating group**.
4. Throughout the study, you will report to the laboratory 4-5 times per week for 8 weeks for hot tub sessions. Additionally, you will participate in 2 experimental days over the course of 8-11 weeks. Experimental Day 1 will occur prior to the 8 weeks hot tub sessions, and day 2 will occur immediately following the 8 weeks of hot tub sessions.

5. During the screening session, we will schedule your initial experimental days (2 sessions), the start of your hot tub sessions, and tentatively schedule all other sessions. We will give you a hard copy of your schedule to take home with you.

Heating (hot tub) Sessions:

1. You will report to the laboratory 4-5 times per week for 8 weeks, for a total of 36 sessions. Each session will take approximately 2-2.5 hours. You will be asked to bring a swimsuit to wear during the session. If you do not have one, we will provide one for you to use for the duration of the 8 weeks. No other subjects will use the same swimsuit.
2. You will be asked to provide a urine sample so that we can ensure you are properly hydrated before undergoing heat stress. If you are dehydrated, we will give you 5mL/kg body weight (about half a normal sized 20oz bottle of Gatorade®) of fluids to drink prior to getting in the hot tub.
3. Your nude body weight will be measured by a member of the same sex prior to getting in the hot tub. You will stand behind a privacy screen while this measurement is taken. Your nude body weight will also be measured following heat stress. This will be done so that we can quantify the volume of sweat you lose while in the tub.
4. You will be instrumented with a Polar® heart rate monitor chest strap so that we can continuously monitor your heart rate throughout the procedure.
5. We will give you a rectal probe labeled with your subject number. It is made of a thin rubber (flexible) material that is inserted 10 cm (approximately 4 inches) past the anal sphincter. The probe will remain in place throughout the entire study session (up to 2.5 hours). The probe has a "tail" that will be connected to an external apparatus. The procedure may be a little uncomfortable at first (during insertion) but it should not be painful at any time. You will be instructed how to self-insert the rectal probe, as well as how to remove it and clean it. If you needed assistance, a lab researcher of the same

sex will help you. Once in place, you may not even feel the probe at all. This technique is widely used and it's considered the "gold standard" procedure for measuring body ("core") temperature.

6. *On the very first and very last heating session (and possibly during one session at about 4wks),* we will place 1 small flexible needle (these are called "intravenous catheters", and are smaller than the lead of a pencil) into a vein near your elbow. The skin will be cleaned before this procedure. This catheter will remain in your vein throughout the heating session. We will take about 40ml of blood, about 2.7 tablespoons, prior to getting into the bathtub, and another 25ml of blood at the end of the heating period so that we can measure various factors in your blood that are affected by the heat exposure. We will remove the catheter after the second blood draw and place a sterile bandage over the site. The vials in which we collect the blood will be coded such that the investigators can determine all samples came from the same subject and the time the sample was taken. No one will be able to determine your identity from the sample.
7. You will then be transferred to a bathtub that will be filled with water at 40°C (104.0°F). The tub is hooked up to a water pump and heater such that water can be circulated and maintained at a desired temperature. You will lay down in the bath tub such that the water level comes up to the level of your collar bone (to the top of your shoulders). You will lay down in this position until your core temperature has reached 38.5°C (101.3°F). This takes approximately 25-35 minutes. At this point, you will sit up such that the water level reaches the middle of your chest (with your shoulders and arms out of the water). You will remain sitting up for another 60 minutes, or until the total time in the hot tub reaches 90 minutes, whichever comes first.
8. We will continuously check in with you throughout the hot tub session. You will be instructed to inform an investigator if you feel any of the following symptoms: light-headedness, dizziness, nausea, headache, or if you feel unbearably warm. If you feel these symptoms, we will have you sit up if you were previously laying down in the bath, or we will have you get out of the bath

if you were previously sitting up. If your body temperature gets too high (above 39.5°C, 103°F), we will have you get out of the bath, even if you feel fine.

9. You will be allowed to drink fluids while in the hot tub.
10. At the end of the heating period, you will get out of the hot tub and sit in a recovery chair. We will continue to monitor your body temperature until your core temperature begins to return back to normal. If your body temperature remains high for too long, or if you feel too hot, dizzy, or nauseous, we will cool you down more quickly with cold packs. We will provide you with a towel to dry off during this time.
11. Once you feel fine, you will remove the rectal probe, clean it, and place it in a location allocated to you.
12. If you lost >1% of your body weight while in the hot tub due to sweating, we will ask you to drink fluids prior to leaving the lab in an amount necessary to return you to no more than a 1% loss of body weight.
13. We will also offer you a light snack before leaving the lab.

Experimental Days 1 and 2 (Skin Studies):

1. You will arrive at Dr. Minson's laboratory in Esslinger Hall at the University of Oregon to participate in the experimental protocol. The testing will take approximately 4-5 hours. The day before the study, we will contact you (either by phone or email depending on your preference) to remind you to refrain from all over-the-counter medications, including vitamins and supplements, for 24 hours, alcohol and caffeine for 12 hours, food for 4 hours, and heavy exercise for 24 hours. Additionally, you will be asked to wear a short-sleeved shirt.
2. Upon arrival at the laboratory, your height and weight and resting blood pressure will be measured. Female subjects will be asked to take a urine pregnancy test. You cannot participate in the study if the pregnancy test is positive, as the study procedures could be harmful to an unborn child.

6. We will place 3 small tubes (these are called "microdialysis fibers", and are about the size of sewing thread) in the skin of your forearm. A small needle will be placed just under the surface of your skin and will exit back out about 1½ inches from where it entered your skin. The small tubes will be placed inside the needle, and the needle will be withdrawn, leaving the small tubes under your skin. These will remain in your skin throughout the rest of the study.
7. We need to wait about 1-2 hours after the small tubes are placed in your skin to let the insertion trauma (redness of your skin around the small tubes) to go away. During this time, a small probe (laser-Doppler probe) will be placed over each area of skin where the small tubes are so that we can measure skin blood flow over the small tube.
8. During the study, we may periodically inflate a small cuff that is placed on your middle finger of one of your hands to measure your blood pressure (Portapres device). We will only inflate this cuff for about 10 minutes at a time. If the cuff becomes uncomfortable, let the investigator know and they will turn it off for a few minutes.
9. Blood pressure will also be measured periodically throughout the study using an inflation cuff on your upper arm.
10. During the protocol we will put some very small doses of drugs through the small tubes in your skin. These drugs will cause the vessels of your skin to either widen or become narrow. You should not feel anything when the drugs are going into your skin. However, it is possible you may feel a slight tingling in the skin where the probe is. You will receive the following drugs:
    a. L-NNA: this stops nitric oxide from being produced and causes the skin vessels to narrow
    b. Tempol: This is a substance that may cause your blood vessels to open.
    c. Sodium nitroprusside: this is a substance that is used to lower blood pressure in patients and causes the skin vessels to open
11. We will heat a small area of your skin with a small heater up to 43.5° Celsius (110 degrees Fahrenheit) to open the vessels in your skin. This is below the

temperature where heating becomes painful (about 113 degrees Fahrenheit) and well below the temperature that may burn your skin (about 117 degrees Fahrenheit). If you think the heater is becoming painful, you need to tell the investigator and the temperature will be lowered.

12. After the study, we will remove the small tubes in your skin and a bandage will be placed over the area of skin where the tubes were placed.
13. Although you will not be allowed food or beverages during the study, you will be given a light snack and fluids to drink before you leave.

Optional Follow-Up Testing:

If you are interested and if you continue to qualify, you may be asked by the investigators to return to the laboratory after you have completed the 8 weeks of hot water immersion. This is so that we can assess how long any changes in your cardiovascular health last. Follow-up testing would take place 2, 4, 6, and/ 8 weeks after your last hot tub session. If you are interested in participating in follow-up testing, you will be asked to give consent and will sign an additional consent form closer to the end of your 8 weeks of hot water immersion. Importantly, if you are not interested in participating in follow-up testing, it does not affect your participation in the primary study, the 8 weeks of hot water immersion. Also please note that all time frames and compensation amounts in this section of the consent form only include the initial 8 weeks of hot water immersion.

If you are interested in being asked later on about participating in follow-up testing, **please initial here: _____**. You can still change your mind at any time.

How long will I be in the study?

You will be in the study for 8-11 weeks. You will participate in one screening session (about 60 min), seven experimental days (a total of up to 25 hours), and 36 heating sessions (up to 2 hours per session).

What are the risks of the study?

1. Heat exposure: There are some risks associated with heat exposure, including: fatigue, light-headedness, muscle cramps, dehydration, and neurological detriments (i.e. heat stroke). However, these symptoms do not typically occur until core temperature rises above 40°C. Your core temperature will be constantly recorded (rectal probe), and you will be removed from the hot bath immediately if either core temperature reaches 39.5°C or you experience any symptoms of heat-related illness. You will be instructed to notify the investigators immediately if you experience any of these symptoms. All symptoms subside upon lowering core temperature. Ice packs will be on hand for rapid cooling if necessary. Additionally, heat exposure may have detrimental effects on a developing fetus in females and` on sperm counts in males. Thus, subjects who are pregnant, trying to conceive, and/or undergoing treatment to increase sperm counts will be excluded from the study.
2. Rectal temperature probes: The use of rectal probes to measure core body temperature, even during exercise, carries minimal risk. The primary risk is of damage to the lining of the rectum; however, this risk is very slight as we use a flexible probe that is designed for this purpose. There is also the risk of infection, either by you not washing your hands properly or exposure to a poorly cleaned probe. The probe has been sterilized before use, and we will instruct you on how to properly clean the probe after each time you use it. The risk of infection is similar to that of having a bowel movement, and is considered minimal (similar to daily experience). There is also the risk of embarrassment. The approach is typically well tolerated by subjects, and the investigative team is professional in regard to how they treat you.
3. Venous blood draws and catheters: There may be some discomfort during the blood draw. Once the catheter is in place, or once the needle is removed, the pain should subside. After the blood draw, the needle will be withdrawn and a sterile dressing will be applied. Any swelling or redness after the study should subside by a few hours after completion of the study. Although the needles are sterile, there is a slight risk of infection at the site where the needle was placed

in your skin. You will be instructed how to keep the area clean for a day or two following the experimental day. The most common complications of inserting a small needle into a vein is a small bruise and pain at the site of the needle location which may last several days after removal of the needle. A small amount of bleeding may occur directly after removal of the catheter. Application of pressure and a gauze dressing will alleviate the bleeding. The maximum amount of blood we will draw across the entire study is 270ml, or about 18 tablespoons, which is well below the volume drawn in a standard blood donation (~450ml). Even so, we will exclude you from the study if you have donated blood within the last two months.

4. Skin microdialysis: There may be some discomfort during the insertion of the small fibers in your skin. Once the needle is in place, the pain should subside. There is also a risk of syncope (fainting) during needle placement. You will be sitting in a reclining chair during the study, which reduces this risk, and you will be asked to inform the investigator if you feel light-headed, nauseous, dizzy, etc. during needle placement. If you do experience any of these symptoms, we will discontinue placing the needles and ensure the symptoms subside. Infusions through the fibers should not be painful, and there should only be minor swelling at the site. At the end of the study, the fibers will be withdrawn and a sterile dressing will be applied. Any swelling or redness after the study should be gone a few hours after completion of the study. Although the small tubes are sterile, there is a slight risk of infection and/or allergic reaction at the sites where the small tubes were placed in your skin. You will be instructed how to keep the area clean for a day or two following the study. If you see any signs of infection (redness, swelling, and/or pain around the sites) or experience some other abnormal reaction at the insertion site following the study, please contact us immediately. We will show you photos of what normal and abnormal healing looks like at the sites. There is a possibility the fibers may break while in your skin or while they are being removed. We remove the fibers in a way such that we can still remove the entire fiber, even if it does

break. However, there is still a slight risk a small part of the fiber could remain in your skin. If this occurs, the piece should be able to work its way out of the skin within a few days (similar to a splinter), and we will follow-up with you to ensure this has happened. If the piece does not work its way out, or if a site seems infected, we will evaluate the site(s) and, if necessary, recommend you seek medical treatment with a healthcare provider.

5. Microdialysis drugs: We will be infusing very small doses of each drug and only into a very small area of your skin. You will not have any systemic (whole body) effects of these drugs, and they will not alter your blood pressure in the small doses given in this study. However, as with any infusions or medications, there is the possibility that you are allergic to the drug and may have an allergic reaction to the drug including changes in blood pressure and difficulty breathing. In the case of an adverse event, the study will be discontinued. Investigators are trained in Advanced Cardiac Life Support and anaphylaxis.
6. Local Skin Heating: The local skin heaters may cause some minor skin discomfort. The goal is to warm the area of skin to a temperature that has been determined to be below the threshold for pain. If the local heating becomes painful, you should tell the investigator and the temperature of the local heater will be lowered. There is a slight risk of burning the skin at this site, so it is important that you tell the investigators of any pain you are feeling. The heating device may be removed at any time if you experience any discomfort.
7. Laser-Doppler Probes: These probes send a small light into your skin. You will not feel anything except the probe touching your skin. There are no major risks associated with this procedure.
8. Emergencies: In the event of an emergency, you will be transported by ambulance to a local emergency facility.

May I participate if I am pregnant or breast-feeding?

No. There is not enough medical information to know what the risks might be to a breast-fed infant or to an unborn child in a woman who takes part in this study. Breast-

feeding mothers are not able to take part in this study. Women who can still become pregnant must have a negative pregnancy test no more than 24 hours before taking part in each experimental day. If the pregnancy test is positive (meaning that you are pregnant), you will no longer be able to take part in the study.

Are there benefits to taking part in this study?

This study will likely not make your health better. This study is being conducted to learn about the effects of chronic heat exposure on cardiovascular health. It is possible the information gathered in this study could be used to better treat patients with elevated cardiovascular risk in the future.

What other choices do I have if I don't take part in this study?

This study is only being done to gather information. You may choose not to take part in this study.

What are the costs of tests and procedures?

You will not need to pay for any tests or procedures that are done just for this research study. You will receive compensation for completed each session in the study as follows: initial screening session, $10; Experimental Days 1-5, $30 each; Experimental Day 6, $50; Experimental Days 7, $40 each; and $10 per heating session completed. If you complete all parts of the study, you will receive **$630**. This money is for the inconvenience and time you spent in this study, and works out to be approximately $10 per hour of participation in experimental days and $10 per heating session. If you start the study but stop before the study has ended, you will get part of this money. The partial amount will be calculated based on which study sessions you completed. You will receive compensation in the form of a check at the end of the study, or if you prefer, approximately every 2 weeks into the study. If you choose to receive compensation every 2 weeks, you will receive an amount corresponding to which study sessions you completed in each two-week time interval. There will be no difference in the total compensation you will receive if you chose to receive compensation at the end of the study versus every 2 weeks.

Please note, compensation from participation in Human Subjects Research studies may be considered taxable income. Compensation amounts are tracked across all studies in which you participate. If compensation totals $600 or more in a calendar year, the University is required to report the income to the IRS. University departments are required to track participant compensation and may contact you to complete a W9 form for tax reporting purposes. Because of this, your name will be associated with participation in a research study. Department and university administrators will have access to this information, but will not have access to research data.

Who can answer my questions?

You may talk to Dr. Christopher Minson or his student, Vienna Brunt, M.S. at any time about any question you have on this study. You may contact Dr. Minson by calling (541) 346-4105, (541) 346-4311 or on his cell phone (541) 953-2231, and Vienna Brunt at (541) 346-4507 or on her cell phone at (541) 968-2635.

What are my rights if I take part in this study?

Taking part in this research study is your decision. You do not have to take part in this study, but if you do, you can stop at any time. Your decision whether or not to participate will not affect your relationship with The University of Oregon.

You do not waive any liability rights for personal injury by signing this form. All forms of medical diagnosis and treatment whether routine or experimental, involve some risk of injury. In spite of all precautions, you might develop medical complications from participating in this study.

The investigators may stop you from taking part in this study at any time if it is in your best interest, if you do not follow the study rules, or if the study is stopped. You will be told of important new findings or any changes in the study or procedures that may happen.

If you experience harm because of the project, you can ask the State of Oregon to pay you. A law called the Oregon Tort Claims Act limits the amount of money you can receive from the State of Oregon if you are harmed. If you have been harmed, there are two University representatives you need to contact. Here

are their addresses and phone numbers:

General Counsel	Research Compliance Services
Office of the President	University of Oregon
University of Oregon	Eugene, OR 97403
Eugene, OR 97403	(541) 346-2510
(541) 346-3082	

What about confidentiality?

Any information that is obtained in connection with this study and that can be identified with you will remain confidential and will be disclosed only with your permission. Subject identities will be kept confidential by assigning you a "subject identification number". The names associated with each subject identification number will be stored on a secure computer with double password protection in Dr. Minson's office and only one list with names and identification numbers will exist. This list will be destroyed after all data has been collected and analyzed and for a period of one year after the results from the study have been published. Only coded specimens and data will exist thereafter.

Your blood will be analyzed for such things as heat shock proteins, hormone concentrations, and cytokines (factors released by cells that affect other cells). Your muscle tissue will be analyzed for changes in DNA (**not genetic testing**), RNA, cell signaling, and changes in cell structure. The researchers may store the information gathered during this study indefinitely.

I have had an opportunity to have my questions answered. I have been given a copy of this form. I agree to take part in this study.

If you have questions regarding your rights as a research subject, contact Research Compliance Services, 5219 University of Oregon, Eugene, OR 97403, 541/346-2510.

Your signature indicates that you have read and understand the information provided above, that you willingly agree to participate, that you may withdraw your consent at any time and discontinue participation without penalty,

that you will receive a copy of this form, and that you are not waiving any legal claims, rights or remedies.

_____ _____

(Date) (Signature of Participant)

_____

(Printed Name of Participant)

_____ _____

(Date) (Signature of Individual Obtaining Consent)

Group 2: Able-bodied thermoneutral water immersion

TITLE: "Chronic heat exposure and cardiovascular health"

INVESTIGATORS: ***Vienna E Brunt, M.S., Dr. Christopher T Minson, and colleagues***

APPROVED BY INSTITUTIONAL REVIEW BOARD: ***February 5, 2014***

This is an important form. Please read it carefully. It tells you what you need to know about this study. If you agree to take part in this research study, you need to sign this form. Your signature means that you have been told about the study and what the risks are. Your signature on this form also means that you want to take part in this study.

Why is this study being done?

Cardiovascular disease is the number one cause of death in the United States. Exercise is a potent means of improving cardiovascular health, but not all patient populations are able to exercise effectively. There is high demand for novel therapies to better manage cardiovascular risk in these patients. Heat exposure can have many beneficial effects on the cardiovascular system. As such, long-term heat exposure (i.e. sitting in a hot tub 4-5x per week) may provide an alternative means to exercise for improving cardiovascular health. This project will assess the benefits of long-term heat exposure on the health of the vasculature and on the cellular pathways that improve vascular health, which is important as the majority

of cardiovascular diseases affect the arteries. We will measure various biomarkers of vascular health before and after 8 weeks of heat exposure in able-bodied individuals and patients with spinal cord injury. SCI patients are a population with elevated cardiovascular risk who have limited exercise capabilities, and who may benefit greatly from chronic heat exposure.

We will address the following questions in this study:

- How does 8 weeks of passive heat exposure affect cardiovascular health, as measured by various biomarkers of cardiovascular function?
- How does 8 weeks of passive heat exposure affect levels of factors circulating in the blood and located within muscle tissue that are important for cardiovascular health?
- Do effects differ between healthy able-bodied individuals and spinal cord injury patients?

What will happen in the study?

1. If you meet all the initial subject criteria (based on initial phone and/or email conversations) and are interested in participating in the study, we will schedule an appointment with you to meet with one of the investigators of the study to discuss the project, to see the laboratory, and to read this form. Additionally, you will fill out a health history form so that we can ensure you are healthy enough to participate in the study. This visit should last about 60 minutes.
2. We will assign you to a subject group. To do this, we will match you to a spinal cord injury patient who is currently enrolled in the study based on your age, sex, and approximate fitness level. You will be assigned to the same group (heating or thermoneutral/control) as that subject. You will be informed of what group you are in prior to reading this form. If you are reading this form, you are in the **able-bodied thermoneutral group.**

3. Throughout the study, you will report to the laboratory 4-5 times per week for thermoneutral water immersion sessions. You will participate in 2 experimental days over the course of 8-11 weeks. Experimental Day 1 will occur prior to the 8 weeks thermoneutral water immersion, and Day 2 will occur immediately following the 8 weeks of thermoneutral water immersion.
4. During the screening session, we will schedule your initial experimental days (3 sessions), the start of your thermoneutral water immersion sessions, and tentatively schedule all other sessions. We will give you a hard copy of your schedule to take home with you.

Thermoneutral Water Immersion Sessions:

1. You will report to the laboratory 4-5 times per week for 8 weeks, for a total of 36 sessions. Each session will take approximately 2-2.5 hours. You will be asked to bring a swimsuit to wear during the session. If you do not have one, we will provide one for you to use for the duration of the 8 weeks. No other subjects will use the same swimsuit.
2. You may be asked to provide a urine sample so that we can ensure you are properly hydrated. If you are dehydrated, we will give you 5mL/kg body weight (about half a normal sized 20oz bottle of Gatorade®) of fluids to drink prior to getting in the tub. You will be able to drink fluids throughout the water immersion period.
3. You will be instrumented with a Polar® heart rate monitor chest strap so that we can continuously monitor your heart rate throughout the procedure.
4. We will give you a rectal probe labeled with your subject number. It is made of a thin rubber (flexible) material that is inserted 10 cm (approximately 4 inches) past the anal sphincter. The probe will remain in place throughout the entire study session (up to 2.5 hours). The probe has a "tail" that will be connected to an external apparatus. The procedure may be a little uncomfortable at first (during insertion) but it should not be painful at anytime. You will be instructed how to self-insert the rectal probe, as well as

how to remove it and clean it. If you needed assistance, a lab researcher of the same sex will help you. Once in place, you may not even feel the probe at all. This technique is widely used and it's considered the "gold standard" procedure for measuring body ("core") temperature.

5. *On the very first and very last heating session (and possibly during one sesson at about 4wks),* we will place 1 small flexible needle (these are called "intravenous catheters", and are smaller than the lead of a pencil) into a vein near your elbow. The skin will be cleaned before this procedure. This catheter will remain in your vein throughout the water immersion session. We will take about 40ml of blood, about 2.7 tablespoons, prior to getting into the bathtub, and another 25ml of blood at the end of the heating period so that we can measure various factors in your blood that may be affected by heat exposure. We will remove the catheter after the second blood draw and place a sterile bandage over the site. The vials in which we collect the blood will be coded such that the investigators can determine all samples came from the same subject and the time the sample was taken. No one will be able to determine your identity from the sample.
6. You will then be transferred to a hot tub. The tub will be filled with water at 36°C (96.8°F). The tub is hooked up to a water pump and heater such that water can be circulated and maintained at a desired temperature. You will lay down in the bath tub such that the water level comes up to the level of your collar bone (to the top of your shoulders). You will lay down in this position for 30 minutes. At this point, you will sit up such that the water level reaches the middle of your chest (with your shoulders and arms out of the water). You will remain sitting up for another 60 minutes.
7. At the end of the water immersion period, you will get out of the tub and sit in a recovery chair. We will give you cool fluids to drink and a towel to dry off, and will offer you a light snack.
8. We will instruct you on how to remove the rectal probe, clean it, and place it in a location allocated to you. You will then be free to leave the lab.

Experimental Days 1 and 7 (Skin Studies):

1. You will arrive at Dr. Minson's laboratory in Esslinger Hall at the University of Oregon to participate in the experimental protocol. The testing will take approximately 4-5 hours. The day before the study, we will contact you (either by phone or email depending on your preference) to remind you to refrain from all over-the-counter medications, including vitamins and supplements, for 24 hours, alcohol and caffeine for 12 hours, food for 4 hours, and heavy exercise for 24 hours. Additionally, you will be asked to wear a short-sleeved shirt.
2. Upon arrival at the laboratory, your height and weight and resting blood pressure will be measured. Female subjects will be asked to take a urine pregnancy test. You cannot participate in the study if the pregnancy test is positive, as the study procedures could be harmful to an unborn child.
3. We will place 3 small tubes (these are called "microdialysis fibers", and are about the size of sewing thread) in the skin of your forearm. A small needle will be placed just under the surface of your skin and will exit back out about 1½ inches from where it entered your skin. The small tubes will be placed inside the needle, and the needle will be withdrawn, leaving the small tubes under your skin. These will remain in your skin throughout the rest of the study.
4. We need to wait about 1-2 hours after the small tubes are placed in your skin to let the insertion trauma (redness of your skin around the small tubes) to go away. During this time, a small probe (laser-Doppler probe) will be placed over each area of skin where the small tubes are so that we can measure skin blood flow over the small tube.
5. During the study, we may periodically inflate a small cuff that is placed on your middle finger of one of your hands to measure your blood pressure (Portapres device). We will only inflate this cuff for about 10 minutes at a time. If the cuff becomes uncomfortable, let the investigator know and they will turn it off for a few minutes.

6. Blood pressure will also be measured periodically throughout the study using an inflation cuff on your upper arm.
7. During the protocol we will put some very small doses of drugs through the small tubes in your skin. These drugs will cause the vessels of your skin to either widen or become narrow. You should not feel anything when the drugs are going into your skin. However, it is possible you may feel a slight tingling in the skin where the probe is. You will receive the following drugs:
    a. L-NNA: this stops nitric oxide from being produced and causes the skin vessels to narrow
    b. Tempol: This is a substance that may cause your blood vessels to open.
    c. Sodium nitroprusside: this is a substance that is used to lower blood pressure in patients and causes the skin vessels to open
8. We will heat a small area of your skin with a small heater up to 43.5° Celsius (110 degrees Fahrenheit) to open the vessels in your skin. This is below the temperature where heating becomes painful (about 113 degrees Fahrenheit) and well below the temperature that may burn your skin (about 117 degrees Fahrenheit). If you think the heater is becoming painful, you need to tell the investigator and the temperature will be lowered.
9. After the study, we will remove the small tubes in your skin and a bandage will be placed over the area of skin where the tubes were placed.

Although you will not be allowed food or beverages during the study, you will be given a light snack and fluids to drink before you leave.

How long will I be in the study?

You will be in the study for 8-11 weeks. You will participate in one screening session (about 60 min), seven experimental days (a total of up to 25 hours), and 36 water immersion sessions (up to 2 hours each).

What are the risks of the study?

1. Rectal temperature probes: The use of rectal probes to measure core body temperature, even during exercise, carries minimal risk. The primary risk is of damage to the lining of the rectum; however, this risk is very slight as we use a flexible probe that is designed for this purpose. There is also the risk of infection, either by you not washing your hands properly or exposure to a poorly cleaned probe. The probe has been sterilized before use, and we will instruct you on how to properly clean the probe after each time you use it. The risk of infection is similar to that of having a bowel movement, and is considered minimal (similar to daily experience). There is also the risk of embarrassment. The approach is typically well tolerated by subjects, and the investigative team is professional in regard to how they will treat you.
2. Venous blood draws and catheters: There may be some discomfort during the blood draw. Once the catheter is in place, or once the needle is removed, the pain should subside. After the blood draw, the needle will be withdrawn and a sterile dressing will be applied. Any swelling or redness after the study should subside by a few hours after completion of the study. Although the needles are sterile, there is a slight risk of infection at the site where the needle was placed in your skin. You will be instructed how to keep the area clean for a day or two following the experimental day. The most common complications of inserting a small needle into a vein is a small bruise and pain at the site of the needle location which may last several days after removal of the needle. A small amount of bleeding may occur directly after removal of the catheter. Application of pressure and a gauze dressing will alleviate the bleeding. The maximum amount of blood we will draw across the entire study is 270ml, or about 18 tablespoons, which is well below the volume drawn in a standard blood donation (~450ml). Even so, we will exclude you from the study if you have donated blood within the last two months.
3. Skin microdialysis: There may be some discomfort during the insertion of the small fibers in your skin. Once the needle is in place, the pain should subside. There is also a risk of syncope (fainting) during needle placement. You will be

sitting in a reclining chair during the study, which reduces this risk, and you will be asked to inform the investigator if you feel light-headed, nauseous, dizzy, etc. during needle placement. If you do experience any of these symptoms, we will discontinue placing the needles and ensure the symptoms subside. Infusions through the fibers should not be painful, and there should only be minor swelling at the site. At the end of the study, the fibers will be withdrawn and a sterile dressing will be applied. Any swelling or redness after the study should be gone a few hours after completion of the study. Although the small tubes are sterile, there is a slight risk of infection and/or allergic reaction at the sites where the small tubes were placed in your skin. You will be instructed how to keep the area clean for a day or two following the study. If you see any signs of infection (redness, swelling, and/or pain around the sites) or experience some other abnormal reaction at the insertion site following the study, please contact us immediately. We will show you photos of what normal and abnormal healing looks like at the sites. There is a possibility the fibers may break while in your skin or while they are being removed. We remove the fibers in a way such that we can still remove the entire fiber, even if it does break. However, there is still a slight risk a small part of the fiber could remain in your skin. If this occurs, the piece should be able to work its way out of the skin within a few days (similar to a splinter), and we will follow-up with you to ensure this has happened. If the piece does not work its way out, or if a site seems infected, we will evaluate the site(s) and, if necessary, recommend you seek medical treatment with a healthcare provider.

4. Microdialysis drugs: We will be infusing very small doses of each drug and only into a very small area of your skin. You will not have any systemic (whole body) effects of these drugs, and they will not alter your blood pressure in the small doses given in this study. However, as with any infusions or medications, there is the possibility that you are allergic to the drug and may have an allergic reaction to the drug including changes in blood pressure and difficulty breathing. In the case of an adverse event, the study will be discontinued.

Investigators are trained in Advanced Cardiac Life Support and anaphylaxis.

5. Local Skin Heating: The local skin heaters may cause some minor skin discomfort. The goal is to warm the area of skin to a temperature that has been determined to be below the threshold for pain. If the local heating becomes painful, you should tell the investigator and the temperature of the local heater will be lowered. There is a slight risk of burning the skin at this site, so it is important that you tell the investigators of any pain you are feeling. The heating device may be removed at any time if you experience any discomfort.
6. Laser-Doppler Probes: These probes send a small light into your skin. You will not feel anything except the probe touching your skin. There are no major risks associated with this procedure.
7. Emergencies: In the event of an emergency, you will be transported by ambulance to a local emergency facility.

May I participate if I am pregnant or breast-feeding?

No. There is not enough medical information to know what the risks might be to a breast-fed infant or to an unborn child in a woman who takes part in this study. Breast-feeding mothers are not able to take part in this study. Women who can still become pregnant must have a negative pregnancy test no more than 24 hours before taking part in each experimental day. If the pregnancy test is positive (meaning that you are pregnant), you will no longer be able to take part in the study.

Are there benefits to taking part in this study?

This study will likely not make your health better. This study is being conducted to learn about the effects of chronic heat exposure on cardiovascular health. It is possible the information gathered in this study could be used to better treat patients with elevated cardiovascular risk in the future.

What other choices do I have if I don't take part in this study?

This study is only being done to gather information. You may choose not to take part in this study.

What are the costs of tests and procedures?

You will not need to pay for any tests or procedures that are done just for this research study. You will receive compensation for completed each session in the study as follows: initial screening session, $10; Experimental Days 1-5, $30 each; Experimental Day 6, $50; Experimental Days 7, $40 each; and $10 per heating session completed. If you complete all parts of the study, you will receive **$630**. This money is for the inconvenience and time you spent in this study, and works out to be approximately $10 per hour of participation in experimental days and $10 per heating session. If you start the study but stop before the study has ended, you will get part of this money. The partial amount will be calculated based on which study sessions you completed. You will receive compensation in the form of a check at the end of the study, or if you prefer, approximately every 2 weeks into the study. If you choose to receive compensation every 2 weeks, you will receive an amount corresponding to which study sessions you completed in each two-week time interval. There will be no difference in the total compensation you will receive if you chose to receive compensation at the end of the study versus every 2 weeks.

Please note, compensation from participation in Human Subjects Research studies may be considered taxable income. Compensation amounts are tracked across all studies in which you participate. If compensation totals $600 or more in a calendar year, the University is required to report the income to the IRS. University departments are required to track participant compensation and may contact you to complete a W9 form for tax reporting purposes. Because of this, your name will be associated with participation in a research study. Department and university administrators will have access to this information, but will not have access to research data.

Who can answer my questions?

You may talk to Dr. Christopher Minson or his student, Vienna Brunt, M.S. at any time about any question you have on this study. You may contact Dr. Minson by calling (541) 346-4105, (541) 346-4311 or on his cell phone (541) 953-2231, and Vienna Brunt at (541) 346-4507 or on her cell phone at (541) 968-2635.

What are my rights if I take part in this study?

Taking part in this research study is your decision. You do not have to take part in this study, but if you do, you can stop at any time. Your decision whether or not to participate will not affect your relationship with The University of Oregon.

You do not waive any liability rights for personal injury by signing this form. All forms of medical diagnosis and treatment whether routine or experimental, involve some risk of injury. In spite of all precautions, you might develop medical complications from participating in this study.

The investigators may stop you from taking part in this study at any time if it is in your best interest, if you do not follow the study rules, or if the study is stopped. You will be told of important new findings or any changes in the study or procedures that may happen.

If you experience harm because of the project, you can ask the State of Oregon to pay you. A law called the Oregon Tort Claims Act limits the amount of money you can receive from the State of Oregon if you are harmed. If you have been harmed, there are two University representatives you need to contact. Here are their addresses and phone numbers:

General Counsel	Research Compliance Services
Office of the President	University of Oregon
University of Oregon	Eugene, OR 97403
Eugene, OR 97403	(541) 346-2510
(541) 346-3082	

What about confidentiality?

Any information that is obtained in connection with this study and that can be identified with you will remain confidential and will be disclosed only with your permission. Subject identities will be kept confidential by assigning you a "subject identification number". The names associated with each subject identification number will be stored on a secure computer with double password protection in Dr. Minson's office and only one list with names and identification numbers will exist. This list will be destroyed after all data has been collected and analyzed and

for a period of one year after the results from the study have been published. Only coded specimens and data will exist thereafter.

Your blood will be analyzed for such things as heat shock proteins, hormone concentrations, and cytokines (factors released by cells that affect other cells). Your muscle tissue will be analyzed for changes in DNA (**not genetic testing**), RNA, cell signaling, and changes in cell structure. The researchers may store the information gathered during this study indefinitely.

I have had an opportunity to have my questions answered. I have been given a copy of this form. I agree to take part in this study.

If you have questions regarding your rights as a research subject, contact Research Compliance Services, 5219 University of Oregon, Eugene, OR 97403, 541/346-2510.

Your signature indicates that you have read and understand the information provided above, that you willingly agree to participate, that you may withdraw your consent at any time and discontinue participation without penalty, that you will receive a copy of this form, and that you are not waiving any legal claims, rights or remedies.

_____ _____

(Date) (Signature of Participant)

_____

(Printed Name of Participant)

_____ _____

(Date) (Signature of Individual Obtaining Consent)

Appendix B: Heating Session Log

Subject ID: _____ **Subject Group:** _____ **Date:** _____ **Time:** _____

Week into HT: _____ **Session #:**_____ **Experimenters:** _____

USG – _____
❒ *If >1.024, drank 5ml/kg before weight*

Chronic Heat Therapy – Heating Session Log

Time (min)	T_{re} (°C)	Water Temp (°C)	HR (bpm)	Subject Position	Notes
0		-		out of the water	
5				fully submerged	
10					
15					
20					
25					
30					
35					
40					
45					
50					
55					
60					
65					
70					
75					
80					
85					
90					
95					
100					
105					

Time when 38.5°C = _____

HR when 38.5°C *(before sitting up/moving)* =

****Continue recording for at least 10 min during recovery and until T_{re} falls below 38.5°C and subject feels fine**

Dry Nude Body weight (kg)	Fluids consumed: bottle weight (kg) **SCI subjects must drink ≥ 1% of BW*						Urine (kg)		
Pre=	#1 Pre =	#2 Pre=	#3 Pre=	#4 Pre=	#5 Pre=		#1 Pre=	#2Pre=	
- Post=	- Post=	- Post=	- Post=	- Post=	- Post=	TOTAL	- Post=	- Post=	TOTAL
Body weight loss=									

Fluids to consume before leaving = [Body weight loss – (pre body weight x 1%)] x 1000ml/kg = _____ml

Sweat loss = body weight loss + fluids consumed – urine = _____kg
Sweat loss as % of pre body wt. = _____%

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