

THE PENNSYLVANIA STATE UNIVERSITY  
SCHREYER HONORS COLLEGE

DEPARTMENT OF BIOLOGY

TOPICAL ILEX APPLICATION INCREASES SKIN BLOOD FLOW

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A thesis  
submitted in partial fulfillment  
of the requirements  
for a baccalaureate degree  
in Biology  
with honors in Biology

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## ABSTRACT

Biofreeze is a commercially available topical analgesic. It is primarily used for its ability to induce cooling sensations mediated by the active ingredient menthol. In addition to menthol, ilex is a putatively inactive ingredient that is purported to act as a skin conditioner and enhance permeability to menthol. Both menthol and ilex are plant extracts, with menthol activating TRPM8 receptors in blood vessel walls and ilex postulated to have dilatory influences on blood vessels. The aim of this study was to examine the separate and combined effects of menthol and ilex on cutaneous vasoreactivity. Cutaneous blood flow was assessed at four sites (1. Biofreeze, 2. Menthol, 3. Ilex, 4. Control) using reactive hyperemia (RH) (5 minute occlusion) and local heating to induce endothelium derived hyperpolarizing factor (EDHF)-mediated and nitric oxide (NO)-mediated vasodilation, respectively. Skin blood flow (SkBF) was measured using laser speckle contrast imaging (moorFLPI) on the forearm skin. For the reactive hyperemia protocol the total hyperemic response (THR = AUC - [(baseline SkBF as % maximal cutaneous vascular conductance  $CVC_{max}$ ) x duration of hyperemic response in s]) was calculated through integration over the hyperemic response above occlusion blood flow and normalized to cutaneous vascular conductance ( $CVC = Flux/MAP$ ) and expressed as a percentage of maximal CVC (% $CVC_{max}$ ). % $CVC_{max}$  was calculated and analyzed for the local heating protocols. All protocols were repeated on the opposite arm after topical 5% lidocaine application (LMX 5 cream) to inhibit sensory nerves. Baseline skin blood flow was increased in the Biofreeze and ilex gel sites ( $P < 0.01$ ) with no significant changes at the menthol site. Similarly, Ilex application increased the local heating plateau ( $P = 0.04$ ). The THR increased with the application of ilex gel ( $P < 0.01$ ) and Biofreeze ( $P < 0.01$ ) (217% increase over placebo). Skin blood flow with Biofreeze and ilex gels after lidocaine application was partially attenuated (26% and 47% decrease respectively), yet continued to be elevated over menthol and placebo sites ( $P < 0.01$ ). These results suggest that

menthol stimulates cold sensation yet has little effect upon cutaneous vasomotor tone, whereas the ilex increases skin blood flow likely through EDHF and NO-dependent mechanisms.

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Dr. James Marden was a great Honors Academic Advisor and I would like to thank him for helping me through the maze of undergraduate study.

## Chapter 1

### INTRODUCTION

Commercially available menthol based gels, including Biofreeze, are used for pain relief by inducing a cooling sensation <sup>[1][2]</sup>. In many cases, the application is intended to substitute for ice or cold water cryotherapy <sup>[2]</sup>. The pain suppressing effects of topically applied analgesics with menthol are mediated through the activation of cold receptors. However, in addition to menthol, many topical analgesics contain other ingredients acting as skin conditioners and permeabilizing agents. These additional ingredients may also alter blood flow properties.

The two primary active ingredients of Biofreeze are menthol and ilex, both derived from plant sources, species of mint and holly respectively. Menthol has a long history of use for its ability to induce a cooling sensation <sup>[1]</sup> through activation of transient receptor potential melastatin 8 (TRPM8) receptors on sensory nerves. TRPM8 receptors are activated at a threshold of 28.4°C, with falling temperature accompanied by steady increases in ion channel activity <sup>[1][3]</sup>. In addition to being located on sensory nerves, TRPM8 receptors are also located in the arterial vascular smooth muscle and may alter vasomotor tone during temperature changes <sup>[3]</sup>. The effects of topical menthol application on the vasculature have yielded mixed results, with some studies reporting diminished brachial artery blood flow while others showed increases in cutaneous blood flow <sup>[2][4]</sup>. However, to date no studies have examined the separate and combined effects of Ilex on skin blood flow. The compositional xanthines and flavonoids present in ilex could potentially have a vasodilator effect in the skin <sup>[5]</sup>. Further, these compounds have been shown to improve endothelial function and vascular responsiveness in animal models <sup>[6][7]</sup>.

The purpose of this study was to examine the effects of menthol and ilex application in Biofreeze gel on cutaneous microvascular blood flow. Additionally, we sought to determine the role of sensory nerve activation in response to menthol and ilex. In order to further clarify the roles of menthol and ilex on specific vascular signaling mechanisms we utilized skin specific stimuli to induce endothelial derived hyperpolarizing factors (EDHF)-mediated vasodilation and endothelial nitric oxide (NO)-dependent vasodilation using reactive hyperemia and local skin heating, respectively <sup>[8][9][10]</sup>. We hypothesized that menthol would increase cutaneous blood flow as a result of activation of TRPM8 receptors in the walls of the blood vessels. Additionally, we hypothesized that ilex would enhance the effects of the menthol and assist in further vasodilation. Finally, we hypothesized that inhibition of sensory nerves would likely diminish the effect of menthol on cutaneous blood flow.

## Chapter 2

### MATERIALS AND METHODS

All protocols were approved by the Penn State Institutional Review Board.

#### Subjects

Ten healthy subjects (5 men, 5 women) were recruited to participate in the study. All subjects were young, ranging from age 21 to 27, and were screened beforehand to ensure good health and no history of cardiovascular disease. Subjects refrained from alcohol and caffeine consumption for 12 hours prior to the experiment.

#### Instruments

Red blood cell flux was measured in the ventral forearm of each subject with the MoorFLPI laser speckle contrast imager. The imager was placed directly over the forearm such that the laser field maximized the on-screen size of the regions of interest. These regions of interest were located on the left and right mid-forearm with supination of the palm.

Each site was approximately 2.5 cm in diameter and covered with an annulus shaped water heater used to clamp local skin temperatures. The heater was applied directly to the skin with an adhesive disk and filled with distilled water. Skin temperature was manipulated through the adjustment of water temperature controlled by the heater's external control panels. Each heater was capped with a transparent plastic disk to prevent water surface interference with the laser speckle contrast.

During the course of the experiment, subjects remained in a sitting or reclined position and blood pressure and electrocardiogram were monitored. Blood pressure was recorded every five minutes and the mean arterial pressure (MAP) was determined in order to normalize the flux values through the calculation of cutaneous vascular conductance ( $CVC = \text{Flux}/\text{MAP}$ ).

## **Protocol**

In a double-blind fashion, four gels were simultaneously applied to the arm under observation: a placebo gel, menthol gel, ilex gel, and a regular Biofreeze gel consisting of both menthol and ilex obtained from the Hygenic Corporation. Half-centimeter distances were maintained between each site. Immediately following gel application, excess gel was dabbed away and local heaters were applied directly on top of the sites. The local heaters were clamped at 34°C for reactive hyperemia and baseline measurements.

*Reactive Hyperemia (RH)* Two reactive hyperemias were performed on the arm of interest, with the moorFLPI measuring all sites simultaneously. Each reactive hyperemia consisted of a five minute baseline recording followed by a five minute occlusion of blood flow to the arm with a blood pressure cuff that was rapidly inflated to suprasystolic pressure. After five minutes, cuff pressure was rapidly decreased and the corresponding reactive hyperemic response was measured. 15 minutes were allowed for recovery before commencement of the next occlusion.

*Local Heating* After the completion of two reactive hyperemic stimulations, the gels for each site and the local heater water were reapplied. A baseline measurement was taken after reapplication and then the local heaters were increased in temperature from 34°C to 42°C at a rate of 0.5°C every 30 seconds. After skin blood flow reached an established plateau (30 min) a five

minute average red blood cell flux at 42°C was obtained, the local site temperature was then increased to 43°C to induce maximum cutaneous vasodilation.

*Sensory nerve blockade* The reactive hyperemia and local heating protocols were repeated with the addition of topical lidocaine application to the regions of interest before specific gel application, on the opposing arm. Lidocaine was applied one hour before the experimental procedure and removed with water immediately prior to gel application. After lidocaine removal the remainder of the protocol was identical to the non-lidocaine protocol.

### **Data Analysis**

The data were collected and stored on an offline computer for later analysis in moorFLPI Review 3.0V software. Baseline flux values were averaged from 3 minute time intervals. Occlusion flux was marked from the time minimum level blood flow was obtained until occlusion release. The reactive hyperemic response was characterized as lasting from the release of the occlusion until the blood flow had returned to the original baseline levels. From these data the average baselines, occlusions, hyperemia durations, and AUCs (Area Under the Curve) were calculated. All intra-subject reactive hyperemia data was averaged between the two repetitions of the test. Cutaneous vascular conductance (CVC) was calculated from the moorFLPI perfusion units as flux/MAP and then normalized in relation to the maximum dilation  $CVC_{max}$  to find the  $\%CVC_{max}$ . The  $CVC_{occlusion}$  was used as a biological zero (0%) and the  $CVC_{max}$  induced through maximum local heating was used as a biological maximum (100%).

The reactive hyperemia was quantified through calculation of the area under the curve of the hyperemic response from the end of occlusion to return to baseline. The integral was taken with the  $\%CVC_{max}$  values over the hyperemic response timeframe.

A repeated measures two-way analysis of variance (ANOVA) test was used to analyze the data. Alpha was set as  $P < 0.05$ .

## Chapter 3

### Results

Subject characteristics are shown in Table 1. All subjects were young and healthy, with no occurrences of obesity or abnormal blood pressure.

**Table 1. Subject Characteristics**

<i>Subject Characteristics</i>	<i>Value (mean±SD)</i>
<b>Men, Women</b>	5, 5
<b>Age (years)</b>	24 ± 3
<b>Height (m)</b>	1.6 ± 0.6
<b>Weight (kg)</b>	74 ± 11
<b>Body Mass Index (BMI) (kg•m<sup>-2</sup>)</b>	23.4 ± 2.7
<b>Mean Arterial Pressure (mmHg)</b>	82 ± 9

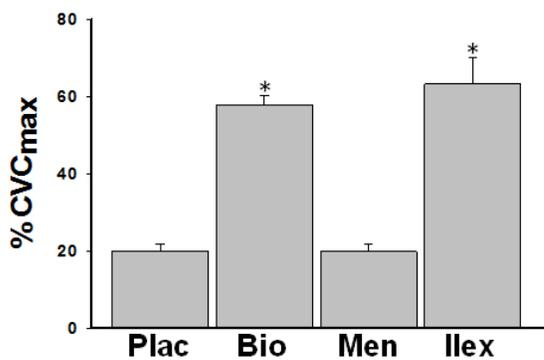
#### **RH Protocol**

The baseline %CVC<sub>max</sub> was increased in both the Biofreeze and ilex gel sites (P<0.01). Figure 1 shows the averaged %CVC<sub>max</sub> at baseline. There was no difference between the Biofreeze and the ilex sites. The menthol gel was indistinguishable from the placebo.

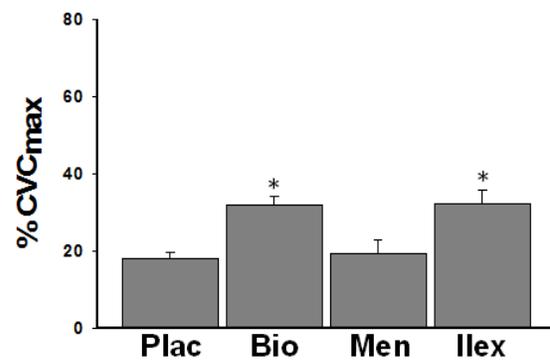
After sensory nerve inhibition skin blood flow was also elevated in the Biofreeze and ilex sites, although they were attenuated compared to sites where the sensory nerves remained

functional. Figure 2 shows the increase in blood flow induced with Biofreeze and ilex. Similar to sites where sensory nerves remained intact, the application of menthol was not different from the placebo.

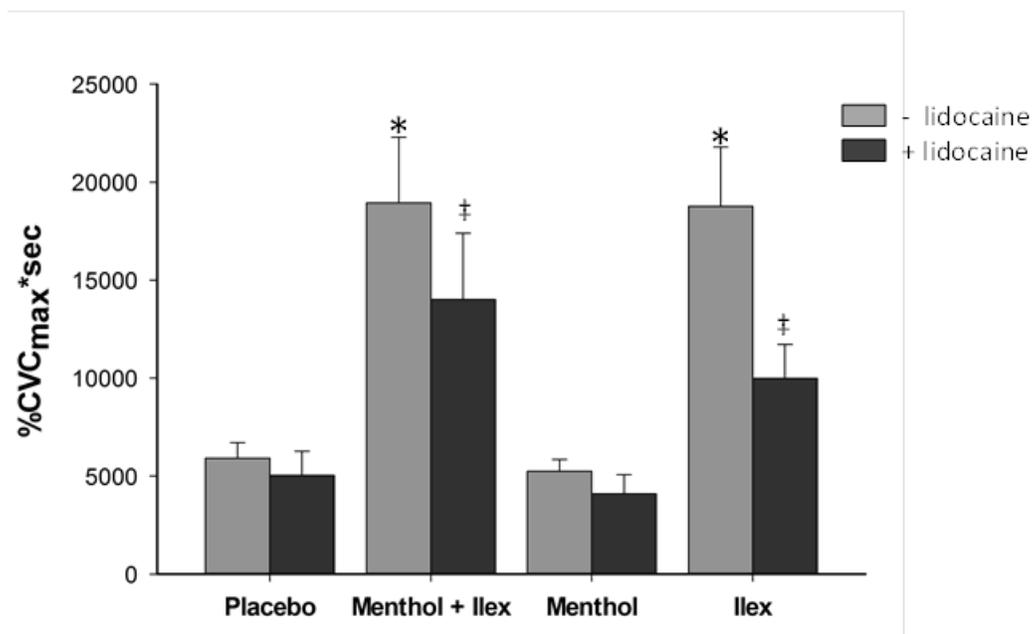
After gel application and RH, the THR was recorded and shown in Figure 3. The Biofreeze ( $P < 0.01$ ) and ilex ( $P < 0.01$ ) sites showed large increases in blood flow over the placebo and menthol sites. With a sensory block, the increased blood flow in the Biofreeze ( $P < 0.02$ ) and ilex ( $P < 0.02$ ) was attenuated yet still greater than the placebo and menthol. The Biofreeze and ilex sites also showed higher amounts of variability between subjects in comparison to the menthol and placebo sites with larger standard error. Once again, the placebo and menthol sites were indistinguishable throughout the experimental section.



**Figure 1.** RH baselines without lidocaine application. The baselines of both RH run during each test were averaged together for each subject individually and then averaged across subjects for the above value. \* designates  $P < .01$  in comparison to placebo.



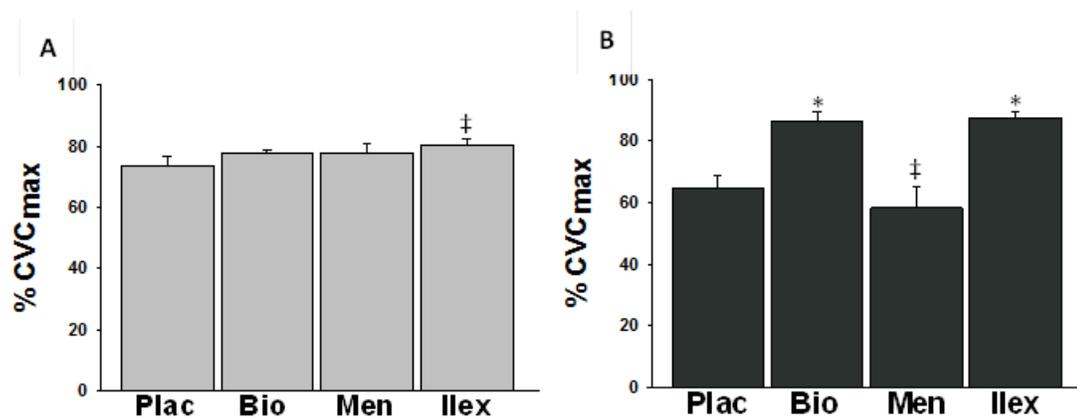
**Figure 2.** RH baselines with lidocaine application. The baselines of both RH run during each test were averaged together for each subject individually and then averaged across subjects for the above value. \* designates  $P < .01$  in comparison to placebo.



**Figure 3.** THR % CVC<sub>max</sub> with and without lidocaine. The THR represents the AUC of the RH response in units of %CVC<sub>max</sub>. Biofreeze and Ilex had a much larger variability between subjects than the placebo and menthol. ‡ denotes .01 < P < .05, \* denotes P < .01 in comparison to placebo.

### Local Heating Protocol

Local heating responses for each site are shown in Figure 4. The non-lidocaine treated sites showed little variation and a small, but significant, increase in %CVC<sub>max</sub> at the illex site compared to placebo (P=0.04). With sensory nerve blockade the blood flow responses were approximately 20% higher than the placebo gel in the illex site (P<0.01), which was greatly diminished by the lidocaine. The menthol site was reduced in comparison to both the non-lidocaine menthol and the lidocaine placebo (P=.04).



**Figure 4.** A. Local heating plateau without lidocaine. B. Local heating plateau with lidocaine. ‡ denotes  $.01 < P < .05$ , \* denotes  $P < .01$  in comparison to placebo.

## **Chapter 4**

### **Discussion**

The illex component of Biofreeze significantly increased skin blood flow, both at baseline and during RH. The increase in blood flow was also present, but attenuated, after the application of lidocaine to remove the contribution of sensory nerves. Contrary to our original hypothesis, application of menthol did not alter skin blood flow under any condition. This suggests that while menthol is capable of inducing a cooling sensation on the skin via activation of TRPM8 receptors, it did not cause changes in cutaneous microvascular tone.

#### **Mechanisms**

These results suggest that illex increases skin blood flow and that this response is partially mediated by sensory nerves, as the application of the lidocaine attenuated vasodilation to some degree. The increased blood flow during the baseline, RH, and local heating suggests that illex induces cutaneous vasodilation through EDHF- and NO-dependent mechanisms.

Contrary to our original hypothesis, menthol did not alter cutaneous vasomotor tone, suggesting that TRPM8 receptors were primarily sensory in nature. They were not directly relaxing the vessel walls, as there was no increase in blood flow during non-lidocaine experimentation, nor were they signaling a cold sensory induced vasoconstrictor pathway, as there was no significant diminishment of blood flow after lidocaine application. It is also possible that the topically applied menthol was unable to diffuse sufficiently to cause TRPM8 receptor activation in the vasculature, or acts via pathways different from those tested in the study. These findings are, however, in contrast with previous research that suggests menthol can cause both

vasodilation and vasoconstriction. This study showed that menthol has little to no effect on vasomotor tone in cutaneous tissue. Menthol may have differing or significantly exaggerated effect on larger vessels not in cutaneous microvascular systems and induce differing effects at high or low concentrations.

The xanthine and flavonoid components of ilex likely induced cutaneous vasodilation. Many xanthine compounds have sympathetic nerve stimulatory actions, such as the stimulant caffeine or theobromine, and would trigger the dilation of vessels in cutaneous tissue<sup>[5][6][7]</sup>. Flavonoid antioxidant characteristics could assist in the vasodilation by diminishing the oxidative radicals from tissue and allowing higher concentrations of EDHFs and NO to be present. Higher concentrations of these compounds would lead to significantly higher dilatory response and basal blood flow.

### **Clinical Application**

The increase in blood flow instigated by the application of Biofreeze could have an impact on the clinically intended use of the gel as a topical analgesic. Because the gel increased blood flow to the application area, it could be problematic for utilization as an alternative to cryogenic therapy because swelling can increase with increased blood flow. The irritation may be decreased due to menthols cooling sensation, but the original problem of swelling could potentially worsen.

The gel possibly has the capability to help with areas of poor circulation or target areas where increased blood flow for therapeutic or higher functionality and health purposes is desired. This would be very helpful in treating the symptoms of diseases such as Raynaud's or peripheral vascular disease, where decreased blood circulation in peripheral tissues is a major issue. The ilex component of Biofreeze could also be used in concert with other topically applied drugs. The ilex

could increase the blood flow to the local area of application in order to increase the rate of absorption into the tissue and blood stream.

### **Limitations**

One limitation in this study was the effectiveness of lidocaine over extended durations of time. The effects of lidocaine typically began to diminish during the second half of the protocol. This problem led to difficulties in accurately determining sensory nerve contributions to vasodilation during local heating.

It must also be taken into account that this study focused on the skin blood flow, and the muscle blood flow was not measured. Further studies are therefore necessary to determine how swelling deep tissue would react.

### **Future Aims**

The findings from this study lead to further questions regarding the possible mechanisms through which *ilex* causes vasodilation. As little is known about the plant extract, further studies are necessary to determine its exact effects on EDHFs and NO. It would also be informative and applicable to test the Biofreeze gel on acral skin to confirm that similar responses are observed in differing types of cutaneous vasculature. For information regarding use under normal application conditions, the gel should be tested without direct contact to water and open exposure to the air. This could lead into studies focusing on the possible therapeutic uses for treating diseases with peripheral vascular constriction.

**Summary**

In summary this study showed the significant vasodilatory effects of ilex at baseline levels as well as during cutaneous RH. The increase in vasodilation also occurred after the application of lidocaine to eliminate sensory effects, suggesting ilex was not primarily a sensory stimulating pathway. Menthol had negligible effects of skin blood flow in almost all portions of the experiment and was not a prominent contributor to cutaneous vasomotor tone.

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# ACADEMIC VITA

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## EDUCATION

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## WORK EXPERIENCE

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*Undergraduate Researcher*

- Conducted research regarding the mechanistic and dilatory effects of menthol and ilex pertaining to microvascular function
- Formulated analytical methods for data quantification and further processing

**University Park, PA**

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### **Emergency Department Mount Nittany Medical Center**

*Volunteer*

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**State College, PA**

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### **Immunology Laboratory University Medical Center Hamburg-Eppendorf**

*Laboratory Intern*

- Assisted in experiment procedure, collect data, and studied relevant research
- Proposed solutions to problems that arose during experimentation

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### **Mount Nittany Medical Center**

*MEDITECH Coach*

- Implemented the new electronic discharge software into the Medical Center and ensured no mistakes were made during discharge
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**State College, PA**

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### **Field Work in Costa Rica**

*Biology 499A; Tropical Field Ecology*

- Surveyed and examined various ecological systems present throughout Costa Rica
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**Costa Rica**

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## LEADERSHIP

### **The Pennsylvania State University: Residence Life**

*Resident Assistant*

- Oversaw residential students on Residence Hall floor to ensure policy adherence, facilitated community activities, and built community among residents

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### **The Pennsylvania State University: Department of Biology**

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- Facilitated the learning of laboratory techniques and styles to students in the introductory biology class
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## ACADEMIC HONORS

- Penn State's Presidents Academic Freshman Award for a 4.0 GPA after the first semester
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