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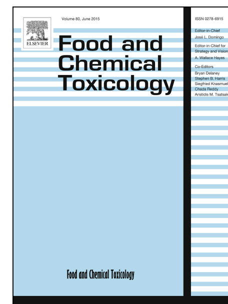

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THE ABSORPTION AND METABOLISM OF A SINGLE L-MENTHOL ORAL VERSUS SKIN ADMINISTRATION: EFFECTS ON THERMOGENESIS AND METABOLIC RATE

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ABSTRACT

We investigated the absorption and metabolism pharmacokinetics of a single L-menthol oral versus skin administration and the effects on human thermogenesis and metabolic rate. Twenty healthy adults were randomly distributed into oral (capsule) and skin (gel) groups and treated with 10 mg·kg^{-1} L-menthol ($ORAL_{MENT}$; $SKIN_{MENT}$) or control (lactose capsule: $ORAL_{CON}$; water application: $SKIN_{CON}$) in a random order on two different days. Levels of serum L-menthol increased similarly in $ORAL_{MENT}$ and $SKIN_{MENT}$ ($p>0.05$). L-menthol glucuronidation was greater in $ORAL_{MENT}$ than $SKIN_{MENT}$ ($p<0.05$). Cutaneous vasoconstriction, rectal temperature and body heat storage showed greater increase following $SKIN_{MENT}$ compared to $ORAL_{MENT}$ and control conditions ($p<0.05$). Metabolic rate increased from baseline by 18% in $SKIN_{MENT}$ and 10% in $ORAL_{MENT}$ and respiratory exchange ratio decreased more in $ORAL_{MENT}$ (5.4%) than $SKIN_{MENT}$ (4.8%) compared to control conditions ($p<0.05$). Levels of plasma adiponectin and leptin as well as heart rate variability were similar to control following either treatment ($p>0.05$). Participants reported no cold, shivering, discomfort, stress or skin irritation. We conclude that a single L-menthol skin administration increased thermogenesis and metabolic rate in humans. These effects are minor following L-menthol oral administration probably due to faster glucuronidation and greater blood menthol glucuronide levels.

KEYWORDS: brown adipose tissue; non-shivering thermogenesis; UCP1; partitional calorimetry; energy consumption; cardiovascular diseases.

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1. INTRODUCTION

Uncoupling protein 1 (UCP1) is the main player in non-shivering thermogenesis (NST) process which takes place in mammal brown and brown-like (beige) adipocytes (Feldmann et al. 2009). Frequent stimulation of UCP1 results in an increase of basal metabolic rate and a reduction of adiposity levels in rodents as the fat energy storage is used for body heat production (Inokuma et al. 2006; Yamashita et al. 2008; Feldmann et al. 2009; Yoneshiro et al. 2013; Valente et al. 2014). Specifically, the oxidation of free fatty acids generates an electrochemical gradient across the mitochondrial inner membrane and when protons bypass the ATP synthase entrance and return to the mitochondrial matrix through UCP1, the energy of the electrochemical gradient is dissipated as heat (Feldmann et al. 2009; Mattson 2010). We and others reported that L-menthol is one of the molecules involved in the activation of UCP1 through the stimulation of the transient receptor potential melastatin 8 ion channel (Tajino et al. 2007; Ma et al. 2012; Valente et al. 2014). This receptor is the principal sensor of thermal stimuli in the peripheral nervous system (Romanovsky 2007; Flouris 2011). It is predominantly located on the cell membrane of sensory neurons while its expression has also been recently discovered on the membrane of brown and white adipocytes (Wang and Woolf 2005; Bautista et al. 2007; Colburn et al. 2007; Dhaka et al. 2007; Tajino et al. 2011; Ma et al. 2012; Rossato et al. 2014). Once in the body, L-menthol stimulates its receptor and induces a reversible rise in the intracellular Ca^{2+} with a subsequent protein kinase A phosphorylation in brown adipocytes that leads to UCP1 activation (Ma et al. 2012). This effect on Ca^{2+} current and UCP1 activation is stereochemically selective as D-menthol is half as active as its stereoisomer L-menthol (Wright et al. 1998).

A single application of L-menthol to the skin of the whole trunk in mice induces heat production and a rise in core temperature through skin vasoconstriction, muscle shivering, and NST activity (Tajino et al. 2007). Moreover, a 7-month dietary L-menthol

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administration in mice increases core temperature, UCP1 expression in brown adipose tissue (BAT), NST activity and insulin sensitivity leading to attenuated body weight gain (Ma et al. 2012).

Similarly to rodents, a single skin L-menthol application in humans evokes cold sensation and cutaneous vasoconstriction (Yosipovitch et al. 1996; Olive et al. 2010; Topp et al. 2011; Gillis et al. 2015). A single oral administration of L-menthol, however, does not influence blood pressure or skin temperature (Gelal et al. 1999). Pharmacokinetics results show that, when taken orally, L-menthol is rapidly absorbed from the small intestine and extracted in the urine predominantly (~65%) as menthol glucuronide (Hiki et al. 2011). These findings suggest that the glucuronidation process which leads to low levels of L-menthol in the blood, may attenuate the aforementioned physiological effect upon oral L-menthol administration. Interestingly, the recent discovery of BAT and the white to brown-like transdifferentiation process in adult humans has provoked a plethora of theories and scientific experiments on its metabolic capabilities and potential for increasing energy expenditure (van Marken Lichtenbelt et al. 2009; Virtanen et al. 2009; Muzik et al. 2012; Rossato et al. 2014; Dinas et al. 2015; Flouris and Dinas 2015). Indeed, a recent in vitro study on human white adipocytes reported that L-menthol induces white to brown-like adipocyte transdifferentiation, stimulates UCP1 expression in brown-like adipocytes, and increases glucose uptake and heat production (Rossato et al. 2014). These findings emphasize the requirement of in vivo studies to confirm L-menthol as a potential molecule involved in controlling energy metabolism in humans. However, no physiological or clinical data are available on the effect of L-menthol on NST and energy expenditure in humans as well as on the comparison between oral and skin L-menthol administration in eliciting these effects.

The purpose of the present study was to compare, for the first time, the absorption and metabolism pharmacokinetics of a single L-menthol oral versus skin

administration and investigate their effects on thermogenesis and metabolic rate in healthy men and women. Based on previous animal studies assessing the effects of a single skin and daily oral L-menthol administration on NST and metabolism (Yosipovitch et al. 1996; Gelal et al. 1999; Tajino et al. 2007; Olive et al. 2010; Topp et al. 2011; Ma et al. 2012; Rossato et al. 2014; Valente et al. 2014) as well as considering the increase in human cutaneous vasoconstriction and rectal temperature upon a single skin L-menthol administration, we hypothesized that both oral and skin L-menthol administration would increase thermogenesis and metabolic rate in humans. Moreover, considering a limited contribution of the skin to the body's total phase II drug metabolism in humans (Manevski et al. 2015), L-menthol transdermal absorption may attenuate and/or delay the glucuronidation process leading to the entrance of a greater amount of L-menthol into the bloodstream compared to gastrointestinal absorption (Gelal et al. 1999; Hiki et al. 2011). Therefore, we also hypothesized that the skin administration would be more potent at eliciting these effects.

2. METHODS

2.1 Participants

The study conformed to the standards set by the Declaration of Helsinki and was approved by the University of Thessaly Department of Exercise Science Ethics Committee. A minimum required sample size of <5 participants per group was determined (statistical power: 0.95; α error probability: 0.05) based on plasma menthol glucuronide concentration observed before (17.8 ± 13.9 ng/ml) and 1 hour after (5617.5 ± 1918.1 ng/ml) a single oral L-menthol administration in a previously-published study (Hiki et al. 2011). Thus, 10 male and 10 female healthy volunteers [25.3 ± 6.2 years, 23.8 ± 3.0 kg·m^2 body mass index (BMI), 20.2 ± 9.9 body fat percentage (mean ± standard deviation)] were recruited for this study. All volunteers were recreationally

active individuals and none of them were competitive athletes. Exclusion criteria included smoking, history or presence of metabolic, cardiac or pulmonary disease, thyroid or thermoregulatory disorders, use of any medication or oral contraception and pregnancy for females. Participants were informed about all experimental procedures, associated risks and discomforts and were asked to provide written informed consent. Female participants were tested during the early follicular phase (days 1-6) of their menstrual cycle.

Volunteers were randomly placed into the oral (ORAL) or skin (SKIN) administration group, each consisting of 10 participants. The study was conducted between June 2013 and June 2014 and participants of the two groups (ORAL and SKIN) were randomly allocated among the four seasons and tested for both control and L-menthol administration within 4-6 days. The randomization of the participants into the two groups as well as among seasons was performed using a random allocation algorithm incorporated in the SPSS Statistics Software (version 19; SPSS Inc., Chicago, IL, USA) in order to eliminate the eventual effects of adaptation/stress to the experimental procedures as well as to season temperature on the obtained results. No differences in age (ORAL: 26.2 ± 8.0 years; SKIN: 24.4 ± 3.9 years), body weight (ORAL: 65.9 ± 11.9 Kg; SKIN: 77.0 ± 11.3 Kg), height (ORAL: 1.7 ± 0.1 m; SKIN: 1.8 ± 0.1 m), BMI (ORAL: 22.6 ± 2.1 Kg·m^2; SKIN: 25 ± 3.4 Kg·m^2), or body fat percentage (ORAL: 17.8 ± 6.8 %; SKIN: 22.6 ± 12.1 %), were detected between the two groups (mean ± SD, $p>0.05$). Participants in the ORAL group were administered L-menthol ($ORAL_{MENT}$) via a capsule containing 10 mg·kg^{-1} body weight of L-menthol crystals (natural L-menthol FU-BP-USP; A.C.E.F. spa, Fiorenzuola d'Arda, Italy). This represents the highest daily orally dose that has been safely administrated to humans (Eisenberg et al. 1955). Participants in the SKIN group were administered L-menthol ($SKIN_{MENT}$) by applying a gel on skin surface of the neck and right arm and leg containing the same

relative amount of L-menthol crystals (i.e., 10 mg·kg^{-1} body weight). The L-menthol gel was formulated according to previously published procedures (Kounalakis et al. 2010) by mixing 10 mL of warm water (~40℃) and crystals of L-menthol for five minutes immediately prior to application. Both groups also completed a control treatment that consisted of a capsule containing lactose for the ORAL group ($ORAL_{CON}$) and warm water (~40℃) application for the SKIN ($SKIN_{CON}$). The administration of L-menthol or control was conducted in a random order on two different days separated by at least 72 hours. All measurements (described in the following paragraphs) were conducted by the same trained researchers using identical pre-calibrated equipment. Double-blind procedures were adopted only in the ORAL group. Due to the strong smell of menthol, a double-blind design was not possible in the SKIN group.

2.2 Experimental procedures

For each participant, the dietary intakes of the day before each administration (i.e., control or L-menthol oral administration as well as control or L-menthol skin administration) were similar. Indeed, on the day before to the first administration (i.e., control or L-menthol), participants chose their food consumption recording all the nutrients and the relative amount of their dietary intake using a log as well as their physical activity using a pedometer (Tanita PD-637, Tokyo, Japan). These data were used as a guide for the participants to follow during the day prior to the second administration, and they were asked to adhere to it as much as possible. All participants were asked to not consume any products containing menthol, abstain from alcohol, caffeine, and environmental tobacco smoke, avoid any physical or mental stress and extreme temperature environments, and fast for 12 hours prior to each administration. Moreover, the National Weather Service Website was used to record the environmental

temperature in the surrounding area of the day before each administration (i.e., control or L-menthol) for both groups (ORAL or SKIN).

During the treatment days, participants were asked to report to the laboratory (which was maintained at 25.1 ± 0.9 ℃ and 35.8 ± 3 .4 % relative humidity during the entire assessment period) at 08:30 following a 12-hr fast. They were asked to refrain from excessive stressors between wake-up time and arrival at the laboratory. All participants were transported to the laboratory via car and they were asked to wear the same clothing for both treatment days. Food consumption was not permitted during data collection. Although room-temperature water was provided *ad libitum*, Mann-Whitney U test showed that participants in ORAL (151 ± 78.0 g) and SKIN (220 ± 83.6 g) groups consumed the same quantity during treatment days ($p>0.05$).

After the participant's arrival in the lab, body mass and body fat percentage were assessed using a body composition analyser (Tanita BF522W, Tokyo, Japan). Rectal temperature (T_{re}), skin blood flow (SkBF), mean skin temperature (T_{sk}), and body heat storage (S) were assessed to determine the effect of L-menthol administration on thermogenesis, while metabolic rate (M) and respiratory exchange ratio (RER) were measured to determine the effect of L-menthol on metabolism and substrate utilization. Therefore, various sensors for these physiological variables measurements were applied on the participants' body. Specifically, a thin and flexible core temperature thermistor (Mon-A-Therm Core, Mallinkrodt Medical, St Louis, USA) was self-inserted 15 cm beyond the anal sphincter to measure T_{re} every 8 sec using the BioTemp^{TM} (model 3.1, Biomnic Ltd., Greece), as previously described (Hartley et al. 2012). A laser-Doppler velocimetry (PeriFlux System 5000, main control unit; PF5010 LDPM, function unit; Perimed, Stockholm, Sweden) was applied on the epidermis at the distal end of the left first toe (i.e. hallux) to measure SkBF, using previous procedures (Flouris and Cheung 2009). The probe (PR 407 small straight probe; Perimed) was held in place with a plastic

apparatus (diameter: 5 mm; PH 07-5; Perimed) and double-sided adhesive strips (PF 105-3; Perimed). The T_{sk}, S, M and RER were measured non-invasively using partitional calorimetric techniques. Seven pre-calibrated heat flow sensors incorporating temperature thermistors were placed on the skin surface of the forehead, abdomen, forearm, hand, quadriceps, shin, and foot and held in place with surgical tape (Leukoplast, BSN medical, Hamburg, Germany) and were used to record data every 8 sec using the BioTemp™ (model 3.1, Biomnic Ltd., Greece). Moreover, to assess autonomic modulation, participants were outfitted with a heart rate chest strap and heart rate variability (HRV) was measured through short-range telemetry at 1,000 Hz with a Polar RS800CX (Polar Electro, Kempele, Finland).

At 09:45, participants were asked to sit on a comfortable chair and remain seated for the next 7 hours and 30 min. For all participants, data collection started at 09:45 and the first 15 minutes (i.e., 09:45-10:00) were used as baseline data. The treatment (i.e., L-menthol or control) was always administered at 10:00 which consisted of ingestion of the L-menthol/lactose capsules for participants in ORAL and application of L-menthol gel/water to the back of the neck and the front of the right arm and leg for SKIN participants. Data collection (the first 15 min of every hour) continued for 7 hours and 15 minutes (i.e., from 10:00 until 17:15) following the administration of the treatment. During data collection participants were instructed to remain silent and as calm and still as possible while between measurements, participants remained seated and either were watching a movie or reading a book. Following the final data collection (i.e., 17:00-17:15), all sensors were removed and the participants were discharged.

2.3 Blood biomarkers

Four venous blood samples (~10 mL each) were collected from an antecubital vein at baseline and at 1, 2, and 6 hours following each administration and then separated in

serum-separator and EDTA tubes. The serum-separator-samples were first kept at room temperature for ~20 min before centrifugation at 3000 rpm for 10 min at 4℃ while the EDTA-samples were immediately centrifuged at 3000 rpm for 10 min at 4℃. The serum and plasma supernatants were collected and stored at -80℃ until analysis.

Serum samples were analysed for L-menthol and its primary metabolite, menthol glucuronide that was calculated as the difference in L-menthol concentrations before and after the samples were treated with β-glucuronidase. Specifically, for the determination of free L-menthol, 1 mL of serum was placed in an 8 mL solid phase microextraction vial. One ml of ultrapure water, 0.25 gr of NaCl and 40 ng of internal standard 1,2,3,4-tetrachloronaphthalene (TCN) were added. For the determination of total L-menthol 1 mL of serum, 0.25 gr NaCl, 875 μL of buffer solution pH=6 (Carl Roth GmbH), 25 μL of the β-glucuronidase enzyme (Type HP-2, Helix pomatia, Sigma), 100 μL of ultrapure water and 40 ng of TCN were placed in solid phase microextraction vials (Hiki et al. 2011). Solid phase microextraction vials were sealed with polytetrafluoroethylene/silicon septum caps and placed in a gas chromatography-mass spectrometry tray. Online extraction followed with a polydimethylsiloxane/divinylbenzene type extraction fibre at 50℃ for 30 min with an agitation speed of 250 rpm. After the completion of the absorption process, the fibre tip was inserted in the injection port of the gas chromatography-mass spectrometry, where it remained for 3 min until the complete release of the analytes. Serum samples with no detected levels of L-menthol were used as blank samples, fortified at different concentration levels (0, 1, 2, 5, 10, 20, 40 and 80 ng/mL for free L-menthol and 0, 5, 10, 20, 40, 80, 160, 320, 640 and 1280 ng/mL for total L-menthol) and used as spiked samples for the preparation of the calibration curves. Samples were analysed with a Shimadzu (Kyoto, Japan) QP-2010 gas chromatography-mass spectrometry equipment. The system was equipped with a split/splitless injection inlet and an AOC-5000 robotic autosampler with the appropriate functions to operate in solid

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phase microextraction mode. Gas chromatography-mass spectrometry solution software was used for instrument control and data analysis. Gas chromatography analysis was conducted on a Supelco SLBtm-5ms (Bellefonte PA, USA) capillary column of 30 m length, 0.25 mm i.d, and 0.25 μm film thicknesses. The AOC-5000 solid phase microextraction autosampler was equipped with a syringe with a 65 um polydimethylsiloxane/divinylbenzene Metal Alloy fibre from Supelco (Bellefonte PA, USA). The following conditions were used: Helium with a constant flow rate of 1 mL/min as a carrier gas, inlet temperature 250℃, splitless injection for 1 min, mass spectrometer interface and ion source temperatures were 300℃ and 220℃, respectively. The column temperature was initially held at 65℃ for 5 min, raised at 10℃·min^{-1} to 140℃ and increase at a rate of 30℃·min^{-1} until 310℃ were it remained for 2 min. Under the above conditions the retention times of L-menthol and internal standard were 11.80 and 17.57 min, respectively. The mass spectrometer detector was operated at the selected ion-monitoring mode (SIM). The qualification m/z ions of L-menthol were m/z=71, 81, 95, 123 and 138 (with target ion m/z=95) while the m/z ions for internal standard were 266 and 194 (with target ion 266).

The plasma samples were analysed for adiponectin (Human adiponectin/Acrp30 ELISA, order number RAB0005, analytical sensitivity <25 pg/mL, intra assay <10%, inter assay <12%; Sigma-Aldrich, St Louis, MO 63013, USA) and leptin concentration (Human Leptin ELISA, order number RAB0333, analytical sensitivity <2 pg/mL, intra assay <10%, inter assay <12%; Sigma-Aldrich, St Louis, MO 63013, USA) – being both hormones involved in UCP1 activity (Asano et al. 2013; Qiao et al. 2014), regulation of energy expenditure and glucose and fatty acid breakdown (Giby and Ajith 2014) – using commercially available ELISA kits that were performed according to the instructions of the manufacturer. Briefly, all plasma samples were diluted 30000 times for adiponectin and 20 times for leptin with sample diluent before performing each hormone analysis.

Standard series and samples were added in duplicate to the plate and adiponectin or leptin presented in each sample was bound to the wells by the corresponding immobilized antibody. The wells were washed with a washing solution and biotinylated anti-human adiponectin or leptin antibodies were added. After washing away unbound biotinylated antibody, horseradish peroxidase-conjugated streptavidin was pipetted to the wells. The wells were again washed, a 3,3′,5,5′-tetramethylbenzidine substrate solution was added to the wells and colour developed in proportion to the amount of adiponectin or leptin bound. The stop solution changed the colour from blue to yellow, and the intensity of the colour was measured at a wavelength of 450 nm.

2.4 Thermal comfort and sensation

At the beginning of baseline and each data collection time, participants were asked to indicate a number for thermal comfort and thermal sensation using previously published scales (Gagge et al. 1969). The range of the thermal comfort scale was from 1 through 5, where 1 = “comfortable”, 2 = “slightly uncomfortable”, 3 = “uncomfortable”, 4 = “very uncomfortable”, 5 = “extremely uncomfortable”; participants could also indicate intermediate numbers (i.e., 1.5, 2.5, 3.5, and 4.5). The range of the thermal sensation scale was from 0 through 10, where 0 = “unbearably cold”, 1 = “very cold”, 2 = “cold”, 3 = “cool”, 4 = “slightly cool”, 5 = “neutral”, 6 = “slightly warm”, 7 = “warm”, 8 = “hot”, 9 = “very hot”, 10 = “unbearably hot”. Moreover, during the entire assessment period, participants were frequently asked to report any kind of stress, discomfort, and/or shivering. Moreover, participants in the SKIN group were also constantly inspected for skin irritation.

2.5 Data computations

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Physiological data (i.e., SkBF, T_{sk}, T_{re}, S, M, RER, and HRV indices) were used to calculate 1-min averages for each 15-min data collection time point. Thereafter, these values were used for subsequent computations as described below. Data for total L-menthol and free L-menthol concentrations were used to calculate menthol glucuronide concentration. The serum concentration of total L-menthol represents the total L-menthol absorption, the free L-menthol serum concentration represents the amount of L-menthol in the body, while the serum menthol glucuronide concentration is an indicator of the metabolism of L-menthol.

Partitional calorimetric estimates of the rates of heat storage were calculated according to the conceptual heat balance equation:

$$S = M - W\ (C + R + E_{sk} + C_{res} + E_{res})\ (W\cdot m^{-2})$$

As previously (Nishi 1981), M was calculated as:

$$M = \frac{VO_2 \cdot \frac{\left(\frac{RER - 0.7}{0.3}\right)\cdot e_c + \left(\frac{1 - RER}{0.3}\right)\cdot e_f}{1000} \cdot 1000\ (W \cdot m^{-2})}{60}$$

where VO_2 is the rate of oxygen consumption ($mL\cdot min^{-1}$); RER is the respiratory exchange ratio (non-dimensional); while e_c and e_f are the energetic equivalents of carbohydrate (21.116 $kJ\cdot L^{-1}$ O_2) and fat (19.606 $kJ\cdot L^{-1}$ O_2), respectively. The VO_2 and RER were based on indirect calorimetry data obtained as participants breathed (during data collection time) through a low resistance one-way valve attached to a face mask. Exhaled gases were assessed using a gas analyser (Oxycon Mobile, CareFusion, San Diego, USA) and RER was calculated as the ratio between CO_2 removed from the body and O_2 consumed. External workload (W) was 0 as the participants remained relaxed in a seated position throughout the experiment.

The combined respiratory heat exchange via convection (C_{res}) and evaporation (E_{res}) was calculated as:

$$C_{res} + E_{res} = [0.0014 \cdot H_p \cdot (34 - T_a)] + [0.0173 \cdot H_p \cdot (5.87 - P_a)] \ (W \cdot m^{-2})$$

where H_p is the rate of metabolic heat production calculated as the difference between M and W (in $W \cdot m^{-2}$); T_a is the ambient temperature (°C); and P_a is the ambient vapour pressure in kilopascals (kPa).

Heat exchange via convection (C) and radiation (R) was calculated as:

$$C + R = \frac{T_{sk} - T_o}{R_{cl} + \frac{1}{f_{cl} \cdot h}} \ (W \cdot m^{-2})$$

where: R_{cl} is the thermal resistance of clothing ($m^2 \cdot °C \cdot W^{-1}$) which was assumed to be negligible due to minimal clothing insulation (i.e., ~0.2-0.3 clo) and, in any case, systematic as participants wore the same clothing on both assessment days; f_{cl} is the non-dimensional area-weighted clothing factor (assumed to be 1.0 with minimal clothing); h is the combined heat transfer coefficient ($W \cdot m^{-2} \cdot °C^{-1}$) calculated as the sum of convective (h_c) and radiant (h_r) heat transfer coefficients (see calculation procedures below). The operative temperature (T_o) was estimated as:

$$T_o = \frac{h_c \cdot T_a + h_r \cdot T_r}{h_r + h_c} \ (°C)$$

where T_r is the radiant temperature (°C) assumed to be equal to T_a.

The h_c and h_r were calculated as follows:

$$h_c = 8.3v^{0.6} \ (W \cdot m^{-2} \cdot C^{-1})$$

$$h_r = 4\varepsilon\sigma \frac{A_r}{A_D}\left(273.15 + \frac{T_{cl} + T_r}{2}\right)^3 \ (W \cdot m^{-2} \cdot °C^{-1})$$

where: v is air velocity ($m \cdot s^{-1}$); ε is the area-weighted emissivity of the skin, estimated as 0.95; σ is the Stefan-Boltzmann constant ($5.67 \cdot 10^{-8}$ $W \cdot m^{-2} \cdot °C^{-4}$); $\frac{A_r}{A_D}$ is the effective radiant surface area (non-dimensional), estimated to be 0.7 for a seated individual (Fanger 1970); and T_{cl} is the temperature of the clothing (°C) which was assumed to be

equal to T_{sk}. Evaporative heat loss was assumed to be negligible as no sweating was apparent.

For the HRV analysis, the heart rate monitor signal was transferred to the Polar Precision Performance Software (release 3.00; Polar Electro Oy), and RR intervals (time intervals between consecutive R waves in the electrocardiogram) were analysed in order to assess the modulation of sympathetic and parasympathetic activities as previously described (Flouris and Cheung 2009). Frequency domain, time domain and nonlinear method analysis was performed using HRV software version 1.1 (Finland; Biomedical Signal Analysis Group, Department of Applied Physics, University of Kuopio, Finland 2002) evaluating the RR interval time series automatically obtained from the raw signals (Rajendra Acharya et al. 2006). The frequency domain with the low frequency, high frequency, and low frequency to high frequency ratio variables were analysed to discriminate between sympathetic and parasympathetic contribution to HRV. For the time domain method, both statistical and geometric parameters were considered for the analysis of Mean RR, Mean HR, standard deviation of RR and heart rate, the square root of the mean of squared differences between successive intervals, the percentage of the differences of successive normal-to-normal intervals 50 ms normalized to all differences within the interval, the number of pairs of successive normal-to-normal that differ by more than 50 ms, the HRV triangular index, and the triangular interpolation of normal-to-normal interval histogram. Nonlinear method was used to analyse the standard deviation of fast and long term RR variability, mean line length, maximum line length, recurrence rate, determinism, Shannon Entropy, approximate entropy, sample entropy, Detrended Fluctuation Analysis α 1 and 2, and correlation dimension.

2.6 Statistical analyses

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Environmental temperature in the surrounding area of the day before each administration and all dependent variables (serum L-menthol and menthol glucuronide, plasma adiponectin, leptin and leptin/adiponectin ratio as well as SkBF, T_{sk} T_{re}, S, M, RER, HRV, thermal comfort and thermal sensation) showed a non-normal distribution therefore non-parametric tests were used throughout. Mann-Whitney U and Wilcoxon Signed-Rank tests were used to compare environmental temperatures in the surrounding area between groups for the same treatment (i.e., $ORAL_{MENT}$ vs. $SKIN_{MENT}$ and $ORAL_{CON}$ vs. $SKIN_{CON}$) and between treatments within the same group (i.e., $ORAL_{CON}$ vs. $ORAL_{MENT}$ and $SKIN_{CON}$ vs. $SKIN_{MENT}$), respectively. Moreover, for each dependent variable, Mann–Whitney U tests were used for the comparisons between groups for the same treatment (i.e., $ORAL_{MENT}$ vs. $SKIN_{MENT}$ and $ORAL_{CON}$ vs. $SKIN_{CON}$) at baseline and each assessment hour separately. Wilcoxon Signed-Rank test was used for the comparisons between treatments within the same group (i.e., $ORAL_{CON}$ vs. $ORAL_{MENT}$ and $SKIN_{CON}$ vs. $SKIN_{MENT}$) at baseline and each assessment hour separately as well as to assess dependent variables differences across time (i.e., differences between baseline and each following assessment hour as well as between consecutive assessment hours within each treatment). All analyses were conducted with SPSS Statistics (version 19; SPSS Inc., Chicago, IL, USA). The data are reported as mean ± SEM and p-values < 0.05 were regarded as statistically significant. We did not adjust for multiple comparisons in our study due to the errors and misplaced emphasis associated with such procedures when applied in actual natural observations (Rothman 1990; Perneger 1998; Feise 2002; Rothman 2014).

3. RESULTS

3.1 Environmental temperatures in the surrounding area

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In order to test our primary and secondary hypothesis, we first examined the impact of the environmental temperature in the surrounding area. Mann-Whitney U and Wilcoxon Signed-Rank tests showed that the environmental temperature in the surrounding area on the day before each administration were not significant different ($p>0.05$) between groups for the same treatment (i.e., $ORAL_{MENT}$: 20.5 ± 1.7 ℃ vs. $SKIN_{MENT}$: 16.4 ± 1.8 ℃; $ORAL_{CON}$: 19.8 ± 2.0 ℃ vs. $SKIN_{CON}$ 15.6 ± 1.6 ℃) and between treatments within the same group (i.e., $ORAL_{CON}$: 19.8 ± 2.0 ℃ vs. $ORAL_{MENT}$: 20.5 ± 1.7 ℃; $SKIN_{CON}$: 15.6 ± 1.6 ℃ vs. $SKIN_{MENT}$: 16.4 ± 1.8 ℃).

3.2 Serum L-menthol and menthol glucuronide concentration

Regarding our primary hypothesis, results from Wilcoxon Signed-Rank tests demonstrated that L-menthol and menthol glucuronide concentrations were significantly different between treatments within the same group (i.e., $ORAL_{CON}$ vs. $ORAL_{MENT}$ and $SKIN_{CON}$ vs. $SKIN_{MENT}$) (Figure 1, $p<0.05$). Regarding our secondary hypothesis, results from Mann-Whitney U tests demonstrated that menthol glucuronide concentrations ($p<0.05$) but not L-menthol ($p>0.05$), were significantly different between groups for the same treatment (i.e., $ORAL_{MENT}$ vs. $SKIN_{MENT}$). Specifically, the body absorption of L-menthol (i.e., total L-menthol) was higher in $ORAL_{MENT}$ (3385.9 ± 1681.1 ng/mL) compared to $SKIN_{MENT}$ (214.8 ± 112.7 ng/mL) (Figure 1, $p<0.05$). The changes observed in the amount of L-menthol in the body (i.e., free L-menthol) were similar in $ORAL_{MENT}$ and $SKIN_{MENT}$ ($p>0.05$), showing an increase within 1 hour after treatments (Figure 1, $p<0.05$). Within 1 hour after $ORAL_{MENT}$, menthol glucuronide was ~38.0 times higher than L-menthol (3299.6 ± 1636.3 ng/mL menthol glucuronide and 86.4 ± 44.7 ng/mL L-menthol) (Figure 1). The peak of menthol glucuronide (4201.2 ± 2189.9 ng/mL) was reached 2 hours after $ORAL_{MENT}$ (Figure 1, $p<0.05$). On the other hand, within 1 hour after $SKIN_{MENT}$, menthol glucuronide was ~1.2 times smaller than L-menthol (95.5 ± 69.7

ng/mL menthol glucuronide and 119.4 ± 42.9 ng/mL L-menthol, Figure 1). The peak of menthol glucuronide was reached 6 hours after $SKIN_{MENT}$ (Figure 1, $p<0.05$), showing a concentration ~5.5 times higher than that of L-menthol (223.8 ± 157.0 ng/mL menthol glucuronide and 40.5 ± 10.0 ng/mL L-menthol). However, even at its peak, menthol glucuronide concentration in $SKIN_{MENT}$ was significantly lower than in $ORAL_{MENT}$ (Figure 1, $p<0.05$).

3.3 Thermogenesis and metabolic response

Regarding our primary hypothesis, figure 2 shows that SkBF was reduced ($p<0.05$) within 1 hour following both $ORAL_{MENT}$ (66.5% reduction) and $ORAL_{CON}$ (79.5% reduction); the latter reduction being significantly greater ($p<0.05$). The changes observed in T_{sk} and T_{re} were similar in $ORAL_{MENT}$ and $ORAL_{CON}$ (Figure 2, $p>0.05$). Indeed, after treatments T_{sk} was attenuated by 1% within 1 hour ($p<0.05$) while T_{re} was increased by 0.1% within 3 hours ($p<0.05$).

SkBF was reduced within 15 min (at time 0) following only $SKIN_{MENT}$ (55% reduction; Figure 2, $p<0.05$). Within 1 hour from the treatment, SkBF was reduced in both $SKIN_{MENT}$ (75% reduction) and $SKIN_{CON}$ (49% reduction); the former reduction being significantly greater (Figure 2, $p<0.05$). The changes observed in T_{sk} and T_{re} were more evident in $SKIN_{MENT}$ compared to $SKIN_{CON}$ (Figure 2, $p<0.05$). Specifically, in $SKIN_{MENT}$, T_{sk} was attenuated by 1.1% within 1 hour and by 1.4% within 2 hours ($p<0.05$) after treatment, while T_{re} was increased by 0.2% within 1 hour and by 0.3% within 2 hours ($p<0.05$) after treatment. In contrast, in $SKIN_{CON}$, T_{sk} was attenuated by only 0.8% within 1 hour and by 1.2% within 2 hours ($p<0.05$) after treatment, while T_{re} was decreased by 0.1% within 1 hour ($p<0.05$) after treatment (Figure 2).

Mann-Whitney U tests indicated that S, M, and RER were significantly different between groups for the same treatment (i.e., $ORAL_{MENT}$ vs. $SKIN_{MENT}$ and $ORAL_{CON}$ vs.

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$SKIN_{CON}$; Figure 3, $p<0.05$), while Wilcoxon Signed-Rank tests showed that significant differences between treatments within the same group (i.e., $ORAL_{CON}$ vs. $ORAL_{MENT}$ and $SKIN_{CON}$ vs. $SKIN_{MENT}$) were observed only in RER (Figure 3, $p<0.05$). Specifically, during the first 5 hours from the treatment, the increase observed in S during $ORAL_{MENT}$ was similar to that observed in $ORAL_{CON}$ (Figure 3, $p<0.05$); the former increase being slightly lower ($p>0.05$). Nevertheless, within 6 hours from the treatment, the increase observed in S was more pronounced during $ORAL_{MENT}$ (1000%) compared to $ORAL_{CON}$ (136%) (Figure 3, $p<0.05$). By 7 hours from the treatment, S increased by 943% ($p<0.05$) during the $ORAL_{MENT}$ and by only 83% ($p<0.05$) during the $ORAL_{CON}$. The changes observed in M during the $ORAL_{MENT}$ and the $ORAL_{CON}$ were opposite in trend (Figure 3, $p<0.05$). Specifically, in $ORAL_{MENT}$, M was attenuated by 12% ($p<0.05$) within 1 hour from the treatment and it subsequently demonstrated an upward trend reaching an increase of 10% within 6 hours from the treatment (Figure 3, $p<0.05$). In contrast, in $ORAL_{CON}$, M was significantly increased by 7% ($p<0.05$) within 1 hour from the treatment and it was subsequently attenuated reaching baseline levels within 2 hours from the treatment (Figure 3, $p>0.05$). The RER was reduced within 1 hour after the treatment in both $ORAL_{MENT}$ (5.4% reduction; $p<0.05$) and $ORAL_{CON}$ (2.3% reduction, $p<0.05$); the former reduction being significantly greater (Figure 3, $p<0.05$).

Statistically significant increases in S and M were observed in $SKIN_{MENT}$ and $SKIN_{CON}$; the former increase being significantly greater (Figure 3, $p<0.05$). Specifically, within 2 hours from the treatment, S was increased by 522% ($p<0.05$) in $SKIN_{MENT}$ and by only 110% ($p<0.05$) in $SKIN_{CON}$, while M was increased by 11% ($p<0.05$) in $SKIN_{MENT}$ and by only 3% ($p<0.05$) in $SKIN_{CON}$. Within 7 hours from the treatment, S was increased by 700% ($p<0.05$) in $SKIN_{MENT}$ and by only 160% ($p<0.05$) in $SKIN_{CON}$, while M was increased by 18% ($p<0.05$) in $SKIN_{MENT}$ and by only 6.7% ($p<0.05$) in $SKIN_{CON}$. Within 1 hour after the treatment, the RER was reduced in both $SKIN_{MENT}$ (4.8% reduction,

$p<0.05$) and $SKIN_{CON}$ (7% reduction, $p<0.05$); the latter reduction being significantly greater (Figure 3, $p<0.05$).

3.4 Plasma hormones concentration

Mann-Whitney U tests showed that leptin ($p<0.05$) but not adiponectin or leptin/adiponectin ratio ($p>0.05$), was significantly different between groups for the same treatment. $ORAL_{MENT}$ (5.5 ± 1.2 ng/mL at baseline vs. 3.6 ± 0.5 ng/mL at 1-hour) and $ORAL_{CON}$ (4.5 ± 0.7 ng/mL at baseline vs. 2.7 ± 0.3 ng/mL at 1-hour) resulted in a significant decrease of leptin within 1 hour ($p<0.05$), while $SKIN_{MENT}$ (2.9 ± 1.27 ng/mL at baseline vs. 1 ± 0.3 ng/mL at 6-hours) and $SKIN_{CON}$ (1.4 ± 0.4 ng/mL at baseline vs. 1 ± 0.3 ng/mL at 6-hours) demonstrated significant reductions in leptin within 6 hours ($p<0.05$).

3.5 Autonomic modulation

Results from Mann-Whitney U and Wilcoxon Signed-Rank tests indicated that the analysed HRV indices from the frequency, time and nonlinear domains were not significant different between groups for the same treatment and between treatments within the same group, showing a similar trend in $ORAL_{MENT}$, $ORAL_{CON}$, $SKIN_{MENT}$ and $SKIN_{CON}$ without any significant variation throughout the entire assessment period ($p>0.05$).

3.6 Thermal comfort and sensation response

Mann-Whitney U test indicated that responses in thermal comfort and sensation were significant different between groups for the same treatment (i.e., $ORAL_{MENT}$ vs. $SKIN_{MENT}$ and $ORAL_{CON}$ vs. $SKIN_{CON}$) (Figure 4, $p<0.05$). Thermal comfort response showed significant differences between $ORAL_{MENT}$ and $ORAL_{CON}$ (Figure 4, $p<0.05$). Indeed,

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within 3 hours from the treatment, the participants in $ORAL_{MENT}$ reported being more uncomfortable than in $ORAL_{CON}$ (Figure 4, $p<0.05$). On the other hand, thermal sensation response showed no significant difference between $ORAL_{MENT}$ and $ORAL_{CON}$ (Figure 4, $p>0.05$).

Thermal comfort response showed no significant difference between $SKIN_{MENT}$ and $SKIN_{CON}$ (Figure 4, $p>0.05$). However, immediately after the treatment (at time 0, $p<0.05$) participants in $SKIN_{MENT}$ reported being more uncomfortable than during $SKIN_{CON}$ (Figure 4, $p<0.05$). Within 4 hours following treatment in $SKIN_{MENT}$ the thermal comfort response returned to baseline (i.e., "comfortable"; Figure 4, $p>0.05$). Thermal sensation response showed significant difference between $SKIN_{MENT}$ and $SKIN_{CON}$ (Figure 4, $p<0.05$). Specifically, within 1 hour after the treatment ($p<0.05$), participants in $SKIN_{MENT}$ reported being cooler than in $SKIN_{CON}$ (Figure 4, $p<0.05$).

All participants reported no stress, discomfort or shivering during the entire assessment period. Moreover, participants in $SKIN_{MENT}$ perceived no skin irritation after the treatment administration and for at least 7 days past the test day.

4. DISCUSSION

This is the first study aiming to assess and compare the absorption and metabolism pharmacokinetics of a single L-menthol oral versus skin administration and investigate their effects on thermogenesis and metabolic rate in men and women. We found that a single skin L-menthol administration can increase thermogenesis and metabolic rate in humans while these effects are minor following a single oral L-menthol administration.

Our novel findings show that the administration of L-menthol through the skin (i.e., gel) leads to very low absorption in the body. Indeed, the total L-menthol following $SKIN_{MENT}$ represented 6% of that observed following $ORAL_{MENT}$. Nevertheless, when administered through the skin, the metabolism of L-menthol was very slow, as indicated

by the low menthol glucuronide concentration within 1 and 6 hours following administration. This slow metabolism of L-menthol when administered through the skin may explain the reduced heat loss (as seen through attenuated skin blood flow and mean skin temperature) and augmented thermogenesis (as seen through metabolic rate) which, in turn, lead to increased body heat storage and rectal temperature. Moreover, L-menthol administration through the skin (i.e., gel), but not through the gastrointestinal tract (i.e., capsule), results in a greater RER – corresponding to an increased carbohydrate oxidation compared to the control condition – within 1 hour.

Administration of L-menthol through the gastrointestinal tract leads to a very high absorption in the body. Indeed, the total L-menthol observed following $ORAL_{MENT}$ was ~16 times higher than that observed following $SKIN_{MENT}$. Nevertheless, when administered through the gastrointestinal tract, the metabolism of L-menthol was very fast, as indicated by higher menthol glucuronide concentration within 1 and 2 hours following oral administration compared to the skin. L-menthol levels following oral administration were similar to that following skin administration, therefore the very high menthol glucuronide concentration observed when L-menthol was administered through the gastrointestinal tract may explain the minor effects observed in heat loss attenuation and thermogenesis following administration. Our findings on the high metabolism of L-menthol when administered through the gastrointestinal tract are in line with previous studies (Gelal et al. 1999; Hiki et al. 2011) reporting that L-menthol gastrointestinal absorption results in low (or undetectable) levels of L-menthol and high levels of menthol glucuronide in the blood. Interestingly, we found that the peak of menthol glucuronide concentration in the blood (1-2 hours after the L-menthol capsule ingestion) corresponded to a strong reduction in metabolic rate compared to the control condition and L-menthol gel application, suggesting an inhibitory effect on metabolism by the menthol glucuronide and/or glucuronidation process. Altogether these results suggest

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that L-menthol concentration in the blood should be high enough, without the interference of its glucuronide form, to activate an unknown mechanism which augments carbohydrate oxidation and thermogenesis.

The overall increase in energy expenditure – as measured by the gain in metabolic rate – after the L-menthol gel application is in accordance with the results of a previous in vitro study which reported that L-menthol induces an increase in glucose uptake and heat production in human white adipocytes (Rossato et al. 2014). Moreover, in accordance with previous studies, our analysis shows that a single application of L-menthol gel resulted in a significant decrease in skin blood flow (Yosipovitch et al. 1996; Olive et al. 2010; Topp et al. 2011; Gillis et al. 2015) and mean skin temperature (Yosipovitch et al. 1996; Olive et al. 2010; Topp et al. 2011) as well as in a significant increase in rectal temperature (Gillis et al. 2015). In contrast to our results, the latter study reported no effects of a single L-menthol skin spray solution on mean skin temperature and mean body temperature (Gillis et al. 2015). This may be explained by the lower L-menthol concentration (about 1/3 of that used in our experiment) as well as the thermometrically-derived calculation of mean body temperature used in the study by Gillis et al. (2015).

Our data indicate that L-menthol capsule ingestion caused a decrease in skin blood flow and mean skin temperature, unlike a previous study which reported no such effects after the ingestion of 10 mg L-menthol (Gelal et al. 1999). These contradictory results may be explained by the difference in L-menthol dosage. For instance, in the previous oral administration study, the lower L-menthol dose administrated (which resulted in undetectable blood L-menthol levels due to its rapid conversion in the glucuronide form) could have suppressed the effect on skin blood flow and mean skin temperature (Gelal et al. 1999).

Since BAT activation was not assessed in the present analysis, the observed L-menthol gel application effect in increasing human thermogenesis and metabolic rate could not be certainly attributed to the activation of UCP1 mediated by blood L-menthol as reported in previous animal studies (Tajino et al. 2007; Ma et al. 2012). In our study, participants rested and fasted for the entire assessment period in a thermoneutral environment. Thus, considering the absence of physical activity, diet-induced thermogenesis or shivering, it seems reasonable to suggest that the gain in thermogenesis after L-menthol gel application, may be mediated by non-shivering thermogenesis. Specifically, our results suggest that the increase in body heat storage observed within the first 2 hours after skin L-menthol administration resulted from both the increase in metabolic rate and attenuation in cutaneous vasoconstriction which is due to mainly for the resting and fasting condition in a thermoneutral environment, as similar results were also observed after the control conditions. Thereafter, the further increase in body heat storage was primarily due to an increase in metabolic rate since no further reduction in cutaneous vasoconstriction was observed. Nevertheless, further analysis should clarify the involvement of BAT activity in increasing body temperature and metabolism in humans after a single L-menthol gel application as well as evaluate if a daily application may lead to a reduction of body weight and adiposity levels in humans. Moreover, as the capsule ingestion resulted in a very high L-menthol absorption but the increased menthol glucuronide interfered with the effects of L-menthol on metabolism activation, it would be interesting to use an inhibitory mechanism for the conversion of L-menthol in its glucuronide form and then evaluate the efficacy of ingestible L-menthol on thermogenesis and metabolism.

An animal study reported that daily oral L-menthol administration in mice increases insulin sensitivity (Ma et al. 2012). Moreover, human studies reported that there is high correlation between insulin sensitivity and leptin/adiponectin ratio in both

adults and children with a lower leptin/adiponectin ratio corresponding to higher insulin sensitivity (Hung et al. 2006; Finucane et al. 2009; Oberhauser et al. 2012). The present study showed that a single L-menthol oral or skin administration does not have effect on the levels of leptin, adiponectin or leptin/adiponectin ratio in the blood. Indeed, the decrease of leptin after either L-menthol gel or capsule administration was probably caused by the fasting condition during the entire assessment period which, in turns, resulted in the increase of participants' hunger, as similar results were also observed after the control conditions. In this light, it is worth mentioning that the prolonged fasting could have interfered in part with the L-menthol administration and therefore, besides explaining some of the observed results (i.e., decrease of leptin), may have masked part of L-menthol effects. Thus, further studies should analyse a long term effect after daily L-menthol gel and capsule administration on insulin and carbohydrate metabolism in humans.

Menthol products have shown different healthy physiological effects. They have been successfully used for many decades as a natural analgesic gel for pain (Fang et al. 2008) and as an oral treatment for disinfection (Iscan et al. 2002). Both oral and skin L-menthol administration were very well tolerated by the participants as thermal comfort and sensation ranged from "comfortable" to "slightly uncomfortable" and from "neutral" to "slightly cool", respectively. Moreover, participants reported no discomfort, shivering, skin irritation, or stress (which was evident also from the unchanged HRV indices) throughout the assessment period. A previous study reported that half of the participants sprayed with an L-menthol solution perceived skin irritation, indicating that a cream solution may be more appropriate as well as that there may be large inter-individual variation in the perception of irritation after a single skin L-menthol application (Gillis et al. 2015). The current results showed no apparent detrimental cardiovascular effects (as evidenced by stable heart rate variability recordings) after a single dose of 10 $mg \cdot kg^{-1}$ body weight of

L-menthol which, in contrast, are evident following specific drugs that also boost metabolic rate (Cypess et al. 2012; Vosselman et al. 2012; Carey et al. 2013; Cypess et al. 2015). Finally, it is important to note that very small differences at baseline between treatments which were deemed as statistically significant by our statistical analysis (e.g., a 0.1℃ difference in T_{re} between $ORAL_{MENT}$ and $ORAL_{CON}$) are of no physiological significance as they are within the normal day-to-day variation. Therefore, these differences are not considered in the context of this study's findings. On the other hand, consistent differences in rectal temperature as high as 0.23 ℃ within 2 hours that cannot be explained by factors other than L-menthol treatment are of major physiological significance.

In conclusion, we show that a single administration of L-menthol via a gel cream increased thermogenesis and metabolic rate in humans. These effects are minor following oral L-menthol administration probably due to a faster metabolism of L-menthol (i.e., glucuronidation process) that occurs when the molecule is absorbed through the gastrointestinal tract leading to higher menthol glucuronide concentration following the oral administration compared to the skin. Considering the tolerability of a single L-menthol administration, future chronic studies are necessary to confirm L-menthol as promising candidate treatment for human cardio-metabolic diseases.

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LIST OF FIGURES

Figure 1. Results (mean ± SEM) and comparisons for serum L-menthol and menthol glucuronide levels at baseline (Base) and 1, 2 and 6 hours after oral and skin treatments. Note: * = significant differences from baseline within the same treatment (Wilcoxon Signed-Rank tests; p<0.05); ‡ = significant differences from the previous time-point within the same treatment (Wilcoxon Signed-Rank tests; p<0.05); † = significant differences between control and L-menthol treatments within the same group for the same time-point (Wilcoxon Signed-Rank tests; p<0.05); Ṩ = significant differences between SKIN vs. ORAL group for the same treatment and time-point (Mann-Whitney U tests; p<0.05).

Figure 2: Results (mean ± SEM) and comparisons for skin blood flow [SkBF in perfusion units (PU)], mean skin temperature (T_{sk}), and rectal temperature (T_{re}) at baseline (Base) and for 7 hours after oral and skin treatments. Note: * = significant differences from baseline within the same treatment (Wilcoxon Signed-Rank tests, p<0.05); ‡ = significant differences from the previous time-point within the same treatment (Wilcoxon Signed-Rank tests, p<0.05); † = significant differences between control and L-menthol treatments within the same group for the same time-point (Wilcoxon Signed-Rank tests, p<0.05); Ṩ = significant differences between SKIN vs. ORAL group for the same treatment and time-point (Mann-Whitney U tests, p<0.05).

Figure 3: Results (mean ± SEM) and comparisons for heat storage (S), metabolic rate (M) and respiratory exchange ratio (RER) at baseline (Base) and for 7 hours after oral and skin treatments. Note: * = significant differences from baseline within the same treatment (Wilcoxon Signed-Rank tests; p<0.05); ‡ = significant differences from the

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previous time-point within the same treatment (Wilcoxon Signed-Rank tests; $p<0.05$); † = significant differences between control and L-menthol treatments within the same group for the same time-point (Wilcoxon Signed-Rank tests; $p<0.05$); $ = significant differences between SKIN vs. ORAL group for the same treatment and time-point (Mann-Whitney U tests; $p<0.05$).

Figure 4: Results (mean ± SEM) and comparisons for thermal comfort and sensation at baseline (Base) and for 7 hours after oral and skin treatments. Note: * = significant differences from baseline within the same treatment (Wilcoxon Signed-Rank tests; $p<0.05$); ǂ = significant differences from the previous time-point within the same treatment (Wilcoxon Signed-Rank tests; $p<0.05$); † = significant differences between control and L-menthol treatments within the same group for the same time-point (Wilcoxon Signed-Rank tests; $p<0.05$); $ = significant differences between SKIN vs. ORAL group for the same treatment and time-point (Mann-Whitney U tests; $p<0.05$).

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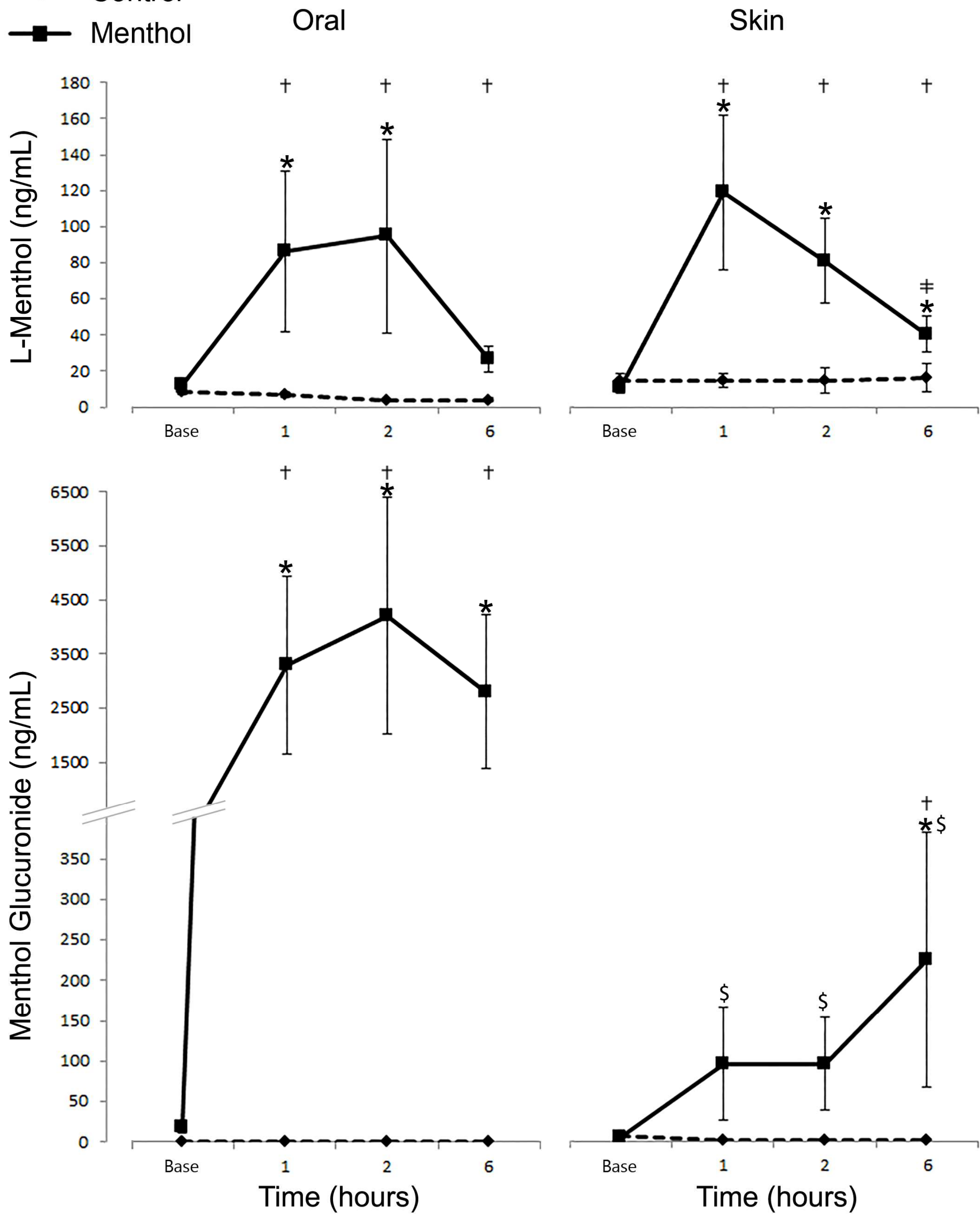
Control
Menthol
Oral
Skin
L-Menthol (ng/mL)
180
160
140
120
100
80
60
40
20
0
Base
1
2
6
Menthol Glucuronide (ng/mL)
6500
5500
4500
3500
2500
1500
350
300
250
200
150
100
50
0
Time (hours)
Time (hours)

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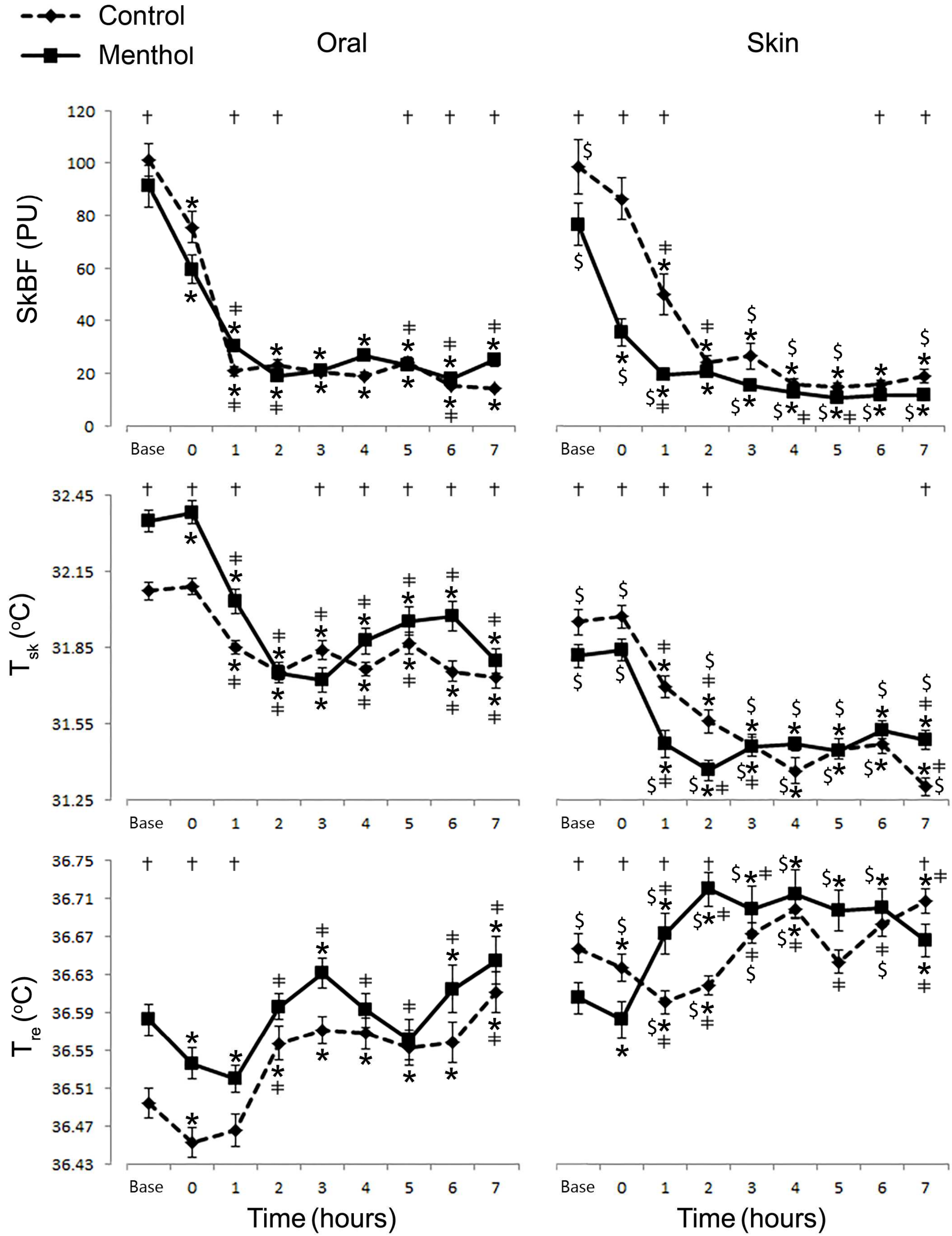
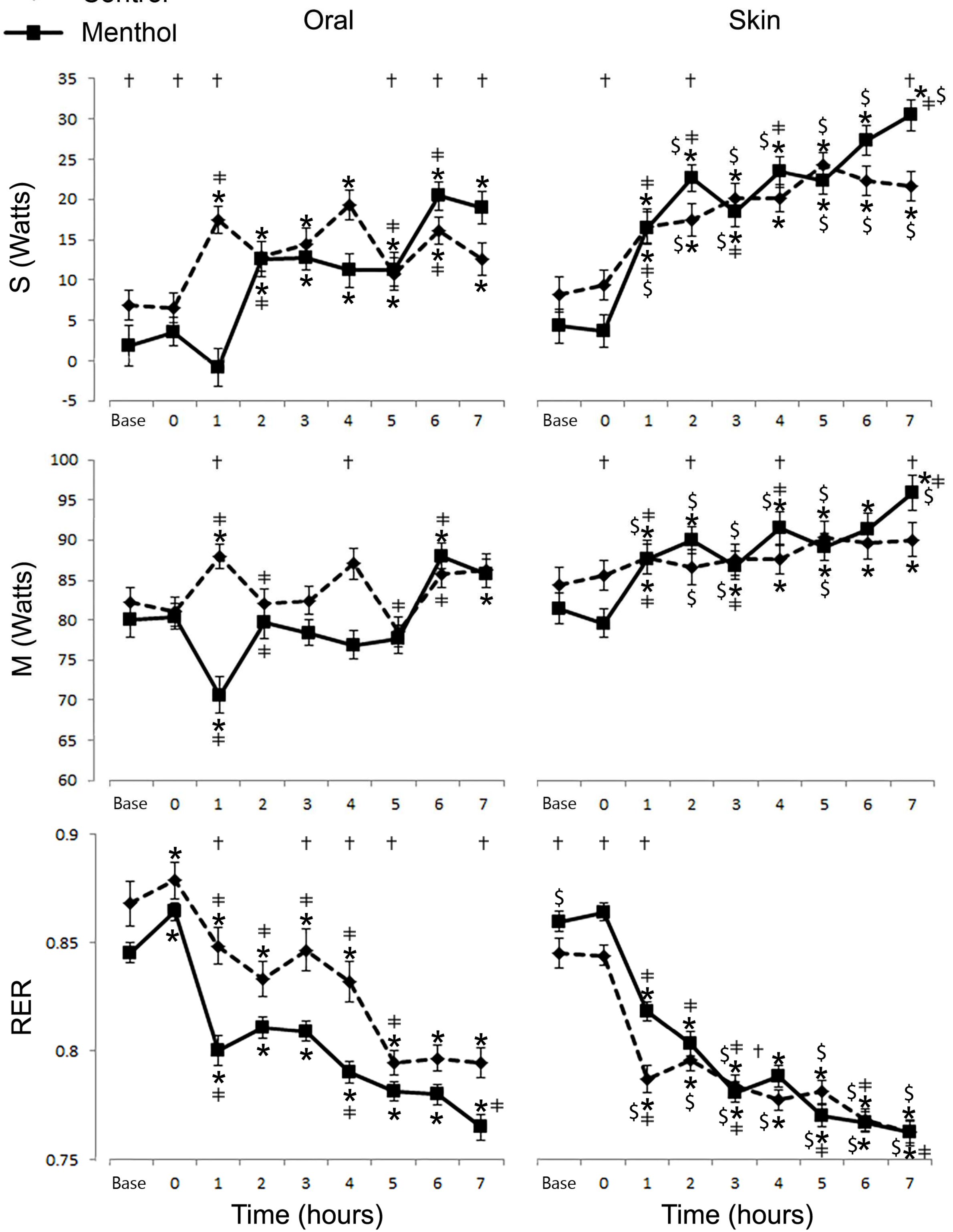
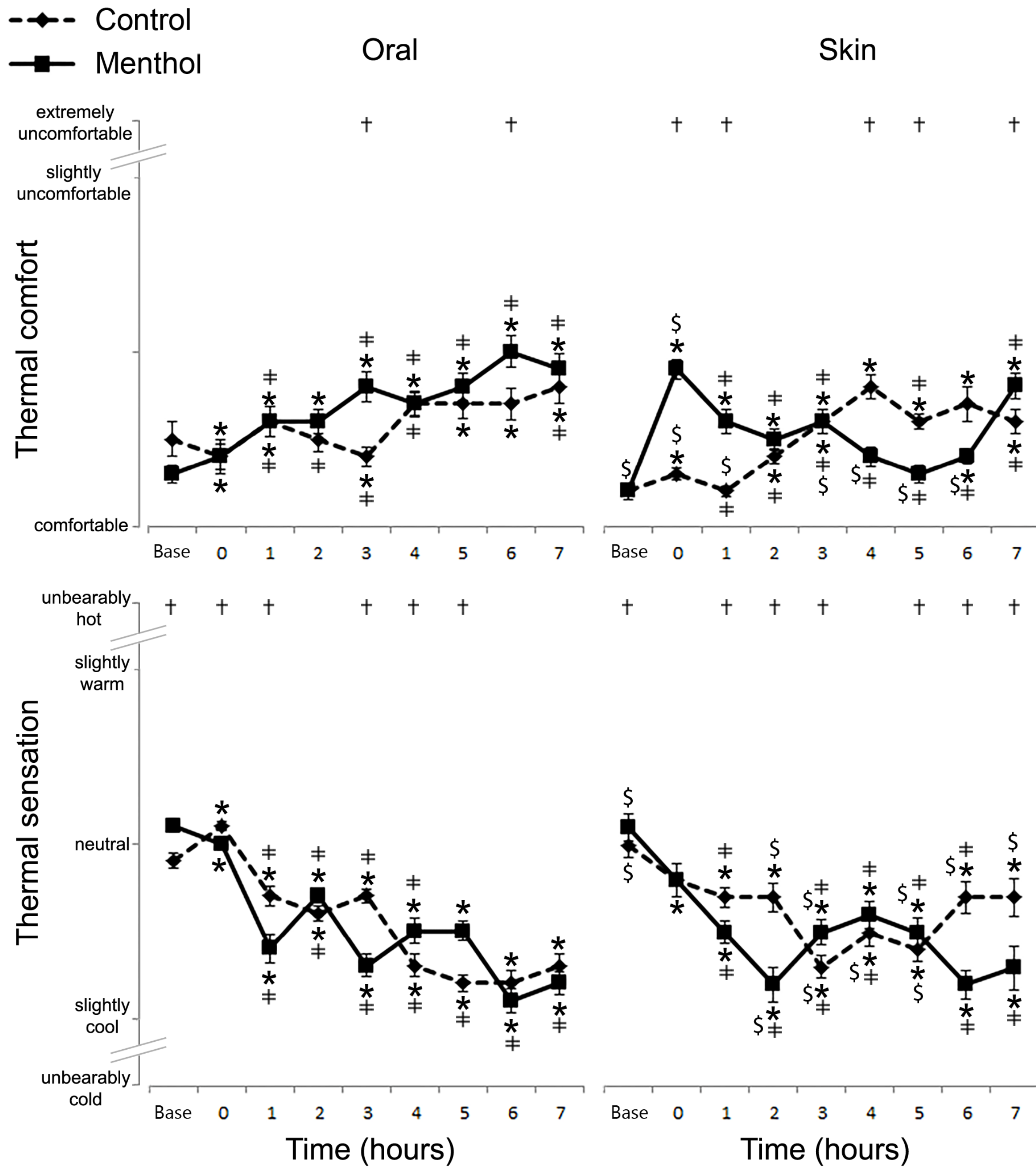




Control
Menthol
Oral
Skin
S (Watts)
M (Watts)
RER
Base
Time (hours)
Time (hours)


Control
Menthol
Oral
Skin
Thermal comfort
extremely uncomfortable
slightly uncomfortable
comfortable
Thermal sensation
unbearably hot
slightly warm
neutral
slightly cool
unbearably cold
Base
0
1
2
3
4
5
6
7
Time (hours)

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Highlights

- A single L-menthol skin administration increased thermogenesis and metabolic rate.
- These effects are minor following oral administration maybe due to glucuronidation.
- L-menthol body absorption was higher after oral administration compared to skin.
- L-menthol levels after oral administration were similar to that following skin.
- Menthol glucuronide levels after oral administration were higher compared to skin.

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