Title

Influence of acute beetroot juice supplementation on cold-induced vasodilation and fingertip rewarming

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Abstract

Purpose: Vasoactive ingredients in beetroot (BR) such as nitrate are known to induce vasodilation in temperate conditions. This study investigated the effect of BR ingestion on cold induced vasodilation (CIVD) and rewarming of finger skin temperature (T_{fing}) during and after hand immersion in cold water.

Methods: Twenty healthy males (mean ± SD; age 22.2±0.7 yrs, height 172.6±6.0 cm, body mass 61.3±11.7 kg) repeated a hand cold water immersion test twice with prior BR or water beverage ingestion (randomised order). They rested for two hours in thermoneutral conditions (27°C, 40% relative humidity) after consuming the beverage, then immersed their non-dominant hand in 8°C water for 30 min. They then rewarmed their hand in the ambient air for 20 min. Skin temperature at seven body sites, T_{fing}, finger skin blood flow ($SkBF_{fing}$), and blood pressure were measured.

Results: During hand immersion parameters of CIVD (T_{fing} and $SkBF_{fing}$) were not different between BR and water conditions although skin temperature gradient from proximal to distal body sites was significantly smaller with BR (P<0.05). During rewarming, $SkBF_{fing}$ and cutaneous vascular conductance were significantly higher with BR than with water (P<0.05). The rewarming speed in T_{fing} and $SkBF_{fing}$ was significantly faster with BR at 15- (BR 1.24±0.22 vs water 1.11±0.26°C/min) and 20-min rewarming (P<0.05). Additionally, individuals with slower rewarming speed with water demonstrated accelerated rewarming with BR supplementation.

Conclusion: BR accelerated rewarming in T_{fing} and $SkBF_{fing}$ after local cold stimulus, whereas, CIVD response during hand cold immersion was not affected by BR ingestion.

Keywords

Nitrate, nitric oxide, rewarming speed, skin blood flow, red beet

Abbreviations

AVA	Arteriovenous anastomoses
CVC	Cutaneous vascular conductance
CIVD	Cold-induced vasodilation
MAP	Mean arterial blood pressure
NFCI	Non-freezing cold injury
NO_3^-	Nitrate
NO_2^-	Nitrite
NO	Nitric oxide
NOS	Nitric oxide synthase
ROS	Reactive oxygen species
SkBF	Skin blood flow
T_{fing}	Finger skin temperature

$T_{\text{pro-dis}}$	Proximal to distal skin temperature difference
T_{subl}	Sublingual temperature
$\bar{T}_{\text{sk}}$	Mean skin temperature

Introduction

In cold environments, cutaneous vasoconstriction is induced for maintaining homeostasis of core body temperature. This vasomotor response for body temperature regulation results in the reduction of skin temperature especially at distal extremities thereby widening the gradient between skin and core and resulting in cooler extremities. Accordingly, the risk of frostbite and non-freezing cold injury in the distal extremities have been reported in the workers in cold environments (e.g. fishery, military, and cold storage workers), especially when they are continuously and longitudinally exposed to cold (Imray et al. 2009; Makinen and Hassi 2009). Additionally, it is well known that performance of manual dexterity using fingers and hands is impaired by the reduction of skin and subcutaneous tissue temperature resulting in impaired motor coordination (Heus et al. 1995; Castellani and Tipton 2015; Wakabayashi et al. 2015), which could be an additional injury risk factor for accidents in the workplace. Thus, wearing thermal protective clothing and gloves are generally recommended for the workers in cold (Castellani et al. 2006; Holmer 2009). However, wearing thick gloves can impair the manual dexterity performance especially when workers conduct technical operations which need fine motor control of their fingers and hands (Brajkovic et al. 2001; Dianat et al. 2012). Therefore, some practically available alternative solutions for keeping warm distal extremities are required for delicate manual work in cold environments in otherwise healthy individuals.

Recently, supplementation with nitrate (NO_3^-) rich beetroot (BR) drink has been considered as an ergogenic aid for enhancing blood circulation and exercise performance, especially in athletes, with promising results (Wylie et al. 2013; Hoon et al. 2013; Dominguez et al. 2017). These studies have concurrently investigated the effect of BR on the blood pressure and vasodilation response but only in thermoneutral or hot environments (Wylie et al. 2013; Hobbs et al. 2013; Amano et al. 2018). Wylie et al. (2013) reported a dose response relationship in reducing the oxygen cost of exercise with no improvement in exercise tolerance above a dose of 140 mL (~8.4 mmol NO_3^-) in a thermoneutral environment. Hobbs et al. (2013), also in a thermoneutral environment, demonstrated increase in the endothelium-independent vasodilation and decreased diastolic blood pressure following beetroot infused bread ingestion. These effects were concurrent with increased plasma and urinary nitrate. Amano et al. (2018) conducted their study in hot conditions (30°C, 50% relative humidity) but only showed changes in mean arterial pressure but not skin blood flow or cutaneous vascular conductance. Compared to studies in warm or thermoneutral environments, fewer studies have investigated the effect of BR on peripheral circulation and distal skin temperature during and after local cold exposure (Eglin et al. 2017; Shepherd et al. 2019; Wickham et al. 2021). This is surprising given that an endothelial-independent NO donor is known to increase vasodilation as demonstrated following glyceryl trinitrate (GTN) ingestion in cold-sensitive individuals (Hope et al. 2014) and topical GTN application in patients with Raynaud's phenomenon (Anderson et al. 2002) suggesting a plausible role for NO in improving peripheral re-warming. Considering the evidence of

cutaneous vasodilation with dietary ingested BR primarily in thermoneutral environments and in persons with cold injury, this intervention could also be applied for maintaining warm finger skin temperature in a cold environment which may also translate to an occupational benefit in improving manual dexterity.

Wickham et al. (2021) investigated the effect of acute BR supplementation on the cold-induced vasodilation (CIVD) in finger skin temperature and blood flow *during* hand cold-water (8°C) immersion in ten healthy males. They found no difference in any CIVD parameters between conditions with BR and NO_3^- depleted placebo drink. Accordingly, they suggested only a minor contribution of nitric oxide (NO) as a mechanism for the CIVD response (Wickham et al. 2021). Shephard et al. (2019) examined the effect of acute and chronic BR supplementation on vasomotor regulation in a cohort of cold-sensitive elderly people (64.3±15.3 yrs) with Raynaud's syndrome (i.e., recurrent transient vasospasm of the fingers and/or toes in response to a cold or stressful stimulus (Wigley 2002)). They reported that both chronic BR supplementation and chronic nitrate depleted BR juice supplementation enhanced skin blood flow (SkBF) during 10-min rewarming phase but in the thumb only following local transient cold stimulus (2-min cold water immersion, 15°C) compared to the baseline no supplementation trial. While, noteworthy there were no other differences between BR and NO_3^- depleted placebo drink. Based on the results, they suggested that some of the vasoactive ingredients in BR, other than NO_3^-, like betanin, quercetin, and chlorogenic acid (Wootton-Beard et al. 2011) might be a factor for enhancing vasodilation after cold stimulus (Shepherd et al. 2019). Importantly these vasoactive substances could be common to both the depleted NO_3^- test supplementation conditions (i.e., the placebo controls) and the BR supplementation conditions used in most studies. Hence, tests using a control condition to effectively separate and distinguish the effects of BR supplementation is warranted. Consistent with this idea, Thompson et al. (2018) compared the physiological effects of BR juice with potassium nitrate (KNO_3) supplementation containing similar amounts of NO_3^- (Thompson et al. 2018). They found lower resting blood pressure with ingestion of BR compared to KNO_3, which suggested ingredients other than NO_3^- in BR might be responsible for improving the bioavailability of NO; yet the study of Thompson et al (2018) was focussed on enhancing sprint interval training performance. Whilst theoretically sound the evidence for the efficacy of BR supplementation in expediting the rewarming of the extremities during and following cold exposure in a healthy population is equivocal although further protocol manipulations are required to explore the putative effects.

With the controls used and findings revealed from previous studies, it remains possible that BR supplementation as a sole ingredient might enhance the CIVD response during local cold exposure and accelerate the subsequent rewarming, in comparison to a control absent of NO_3^- and other vasoactive ingredients (i.e., water ingestion). Accordingly, the purpose of this study is to investigate the effect of acute BR ingestion on CIVD response during hand cold immersion and subsequent rewarming, compared to a control condition (water) in healthy young individuals. It was hypothesized that acute BR supplementation

would enhance the CIVD response during hand cold immersion, and finger skin blood flow and skin temperature rewarming after the immersion. With occupational cold exposure often far longer than the short exposure windows used in prior studies (e.g. Shephard et al. 2019 used 2-min immersion), we also sought to examine an extended period of cold exposure (matching that of Wickham et al. 2021; 30-min) coupled with an extended period of re-warming (i.e., 20-min). Lastly and from a practical perspective, the individual variation in the response to BR ingestion was quantified to enable targeted future intervention toward cold sensitive individuals.

Methods

Participants

The experimental protocol was approved by the IRB of Hokkaido University. All participants were informed of the experimental protocols and gave their written informed consent before participation. Twenty healthy Japanese males living in Sapporo (mean ± standard deviation age: 22.2±0.7 yrs, height: 172.6±6.0 cm, body mass: 61.3±11.7 kg, % body fat: 15.2±5.0%) participated in the experiment. Their percentages of body fat were estimated using bioelectrical impedance (RD-800, TANITA, Japan). All experimental protocols in this study were designed according to the principle of the Helsinki Declaration. Participants were asked to prohibit eating nitrate (NO_3^-) rich foods, e.g. processed meats, green leaf vegetable like Spinach, Chin gin cai, Seaweed, Sayaingen beans (Sobko et al. 2010) on the test day and the day before. Additionally, they fasted for 2 hours before arriving the laboratory and were asked to refrain using mouth rinse on the test day, since the oral bacteria are involved in the reduction of NO_3^- to NO_2^- (Govoni et al. 2008).

Experimental Design

Participants completed a total of two test conditions separated by a minimum of 7 days to enable washing out of the BR effect (Amano et al. 2018; Shepherd et al. 2019). On each occasion hand immersion in to cold water and a rewarming test was completed with prior ingestion of beetroot (BR) or water as a representative control. The order of the test conditions was randomised using crossover design.

Protocol

Participants arrived at the laboratory and changed their clothes to half sleeve shirts, long pants and socks (insulation ~0.6 clo). They then rested in upright sitting position on a chair with their arms on a table in a climatic chamber controlled to thermoneutral conditions (27°C and 40% relative humidity) for 30 min before drinking 140 mL of water or 140 mL of BR (Beet It Sport Pro-Elite Shot, James White Drinks, Ipswich, UK), which were maintained at room temperature (~27°C). The BR drink contains ~12.9 mmol (800 mg) NO_3^- and 0.28 mmol (154 mg) betanin. The concentration of betanin was calculated from absorbance measured with a UV/vis spectrophotometer (U-3310, Hitachi, Japan) using the molar extinction

coefficient ε_{538}=60,000 $M^{-1}cm^{-1}$ (Wyler and Meuer 1979; Kugler et al. 2004). After the beverage ingestion, they continued resting for two hours, which has previously shown to be a sufficient time course to increase plasma NO_2^- after drinking BR containing 4.2 to 16.8 mmol NO_3^- (Wylie et al. 2013). Then, after measuring pre-immersion baseline for the measurement items described below, they immersed their non-dominant hand covered by a waterproof polyethylene glove (12 μm thickness) in 8°C water up to their wrist for 30 min. The water in the tank was stirred and temperature controlled using a thermostat water circulation device (LV-200, Advantec, Japan); water temperature was monitored at the start and end of each immersion. After the 30-min hand water immersion, they removed the glove and rewarmed their hand in lateral position on the table for 20 min.

Measurements

Sublingual temperature (T_{subl}) was measured at baseline, at the end of the hand immersion and rewarming phases using a thermometer (MC-172L, Omron, Japan). Participants were asked to place the tip of the thermometer below the tongue and to close their mouth for 5 minutes until the measurement stabilized. Skin temperature was measured using thermistor probes (ITP082-24, Nikkiso-Therm, Japan) at seven body sites (forehead, chest, forearm, thigh, foot, non-immersed hand and immersed fingertip). The skin temperatures were monitored every second using data loggers (NR543R, Nikkiso-Therm, Japan), and averaged every minute for subsequent data analyses. Mean skin temperature ($\bar{T}_{sk}$) was estimated using a modified Hardy and DuBois' equation (Hardy and Du Bois 1938), as follow:

Equation 1 $$\bar{T}_{sk} = 0.07T_{head} + 0.35T_{chest} + 0.14T_{forearm} + 0.05T_{hand} + 0.32T_{thigh} + 0.07T_{foot}$$

Where: T_{chest} was the selected site for trunk temperature and T_{thigh} included the additional 0.13 weighting ordinarily allocated to T_{legs} (not recorded in the current study) from the original $\bar{T}_{sk}$ formula.

The difference between proximal (average of forehead and chest) and distal (average of hand and foot) skin temperatures ($T_{pro\text{-}dis}$) were calculated as surrogate measure for assessing peripheral blood flow (Rubinstein and Sessler 1990). Since vasoconstriction occurs remarkably in the distal part relative to the proximal part, $T_{pro\text{-}dis}$ well reflects the peripheral vasomotor tone, although there is a limitation that $T_{pro\text{-}dis}$ takes longer time to reach steady state compared to the vasomotor response (Rubinstein and Sessler 1990; House and Tipton 2002). This method has been verified during cooling and rewarming (House and Tipton 2002) and also been used in studies on circadian rhythm as a parameter of distal heat loss (Krauchi et al. 1999). Skin blood flow in the volar side of index finger ($SkBF_{fing}$) was measured by laser Doppler flowmetry (ALF21, ADVANCE, Japan) and sampled using an analogue to digital data converter (Powerlab/16SP, AD Instruments, Australia) and recorded every 1 sec interval using a laptop computer. Arbitrary units (AU) were used for the data of $SkBF_{fing}$. Systolic (*SBP*) and diastolic blood pressure (*DBP*) was measured at the

upper (contralateral) arm using an inflatable cuff and an automated blood pressure monitor (HEM-7430, Omron, Japan) every 5 min during the rewarming phase. Mean arterial blood pressure (*MAP*) was calculated using the following formula:

Equation 2 MAP [mmHg] = ($SBP - DBP$) / 3 + DBP

Cutaneous vascular conductance in finger (CVC_{fing}) was calculated from $SkBF_{fing}$ and *MAP* as follows:

Equation 3 CVC_{fing} [AU/mmHg] = $SkBF_{fing}$ / MAP

Parameters of CIVD were analysed in accordance with (Cheung 2015) where a minimum increase of 0.5°C in T_{fing} was required and the associated change in $SkBF_{fing}$ during the hand cold water immersion was then considered. The onset time of the CIVD, the minimal (T_{min}), the first peak (T_{peak}), and maximal (T_{max}) T_{fing} was detected, then the amplitude from T_{min} to T_{peak} ($T_{peak} - T_{min}$) and mean value of T_{fing} after 5 min to the end of the immersion (mean T_{fing}) were calculated. Additionally, the numbers of CIVD oscillations were counted. These CIVD parameters for $SkBF_{fing}$ were similarly analysed.

Rewarming speed in T_{fing} and $SkBF_{fing}$ was calculated from 1 min to 5, 10, 15, and 20 min after hand immersion, respectively, e.g. T_{fing} rewarming speed in 10 min was calculated as follows:

Equation 4 T_{fing} rewarming speed in 10 min [°C/min] = (T_{fing} at 10 min – T_{fing} at 1 min) / (10 – 1)

Thermal sensation of whole-body and immersed hand was assessed using a 7-points categorical scale (-3: cold, -2: cool, -1: slightly cool, 0: neither, +1: slightly warm, +2: warm, +3: hot) every 5 min during the experiment. Thermal comfort was assessed using a 7-points scale (-3: very uncomfortable, -2: uncomfortable, -1: slightly uncomfortable, 0: neither, +1: slightly comfortable, +2: comfortable, +3: very comfortable) every 5 min. Pain sensation of the immersed hand was assessed using 4-points scale (0: no pain, 1: slightly painful, 2: painful, 3: very painful) every 5 min.

Statistics

Comparisons of datasets from time-course measurements every 5 min were performed using repeated two-way (time × condition) analysis of variance (ANOVA) for each phase of hand cold immersion and rewarming. If Mauchly's sphericity test was not satisfied, the degrees of freedom were adjusted by Greenhouse-Geisser's ε. Partial η^2 (η_p^2) was calculated for assessing effect size for ANOVA where 0.01, 0.06 and 0.14 indicate small, medium and large effect sizes, respectively. Post-hoc test was conducted using a paired Student's *t*-test with multiple comparisons adjustment using Benjamin-Hochberg's false discovery rate (FDR) at time points between water and beetroot conditions. CIVD parameters of T_{fing} and $SkBF_{fing}$ in

two conditions were compared using a paired Student's *t*-test. Pearson's correlation coefficients and 95% confidence interval (CI) for the slope of the regression line were calculated to examine the relationships between rewarming speeds in the two conditions. Wilcoxon signed-rank test was conducted for comparing subjective sensation between conditions. Statistical significance was set at $P<0.05$. Analyses were conducted using a statistical software (IBM SPSS Statistics version 20, IBM). All data are presented as mean values and standard deviation (SD).

Results

Hand cold immersion phase

Time course of T_{fing} and $SkBF_{fing}$ in the immersed hand are shown in **Fig. 1**. A significant main effect of time was detected in T_{fing} ($F_{3.2, 61.1}$=3803.2, η_p^2=0.995, $P<0.001$) and $SkBF_{fing}$ ($F_{2.1, 40.0}$=102.2, η_p^2=0.843, $P<0.001$). Parameters of CIVD in T_{fing} and $SkBF_{fing}$ are presented in **Table 1**. There was no significant difference between conditions in any parameters of CIVD.

Time course of $\bar{T}_{sk}$, T_{hand} on non-immersed body region and difference between proximal and distal skin temperatures ($T_{pro\text{-}dis}$) are shown in **Fig. 2**. During the hand immersion phase, a significant main effect of time was detected in $\bar{T}_{sk}$ ($F_{2.5, 47.5}$=19.6, η_p^2=0.507, $P<0.001$), T_{hand} ($F_{2.1, 39.6}$=65.4, η_p^2=0.775, $P<0.001$), and $T_{pro\text{-}dis}$ ($F_{1.9, 36.6}$=133.3, η_p^2=0.875, $P<0.001$). A significant main effect of condition was detected in T_{hand} ($F_{1, 19}$=6.0, η_p^2=0.239, $P<0.05$) and $T_{pro\text{-}dis}$ ($F_{1, 19}$=6.4, η_p^2=0.253, $P<0.05$). $T_{pro\text{-}dis}$ in BR was significantly smaller than in water condition at 20- and 25-min hand immersion (both $P<0.05$) primarily due to higher temperatures at the distal site. No difference in T_{subl} between water and BR conditions was observed at the baseline (36.60±0.30 and 36.66±0.34°C) and at the end of hand immersion (36.58±0.34 and 36.66±0.33°C). Time course of thermal sensation of the whole-body and immersed hand, thermal comfort and pain sensation of the immersed hand are shown in **Fig. 4**. No difference in these subjective sensations was observed between conditions during hand immersion.

Rewarming phase

Time course of T_{fing} and $SkBF_{fing}$, CVC_{fing} in the immersed hand, and *MAP* during the rewarming phase is shown in **Fig. 3**. A significant main effect of time was detected in T_{fing} ($F_{1.8, 34.0}$=379.3, η_p^2=0.952, $P<0.001$), $SkBF_{fing}$ ($F_{2.3, 43.4}$=55.1, η_p^2=0.744, $P<0.001$), CVC_{fing} ($F_{2.5, 47.9}$=53.8, η_p^2=0.739, $P<0.001$), and *MAP* ($F_{2.9, 54.7}$=4.8, η_p^2=0.202, $P<0.01$). A significant main effect for condition was detected in CVC_{fing} ($F_{1, 19}$=4.4, η_p^2=0.189, $P<0.05$). A significant interaction between time and condition were detected in T_{fing} ($F_{2.3, 44.6}$=5.5, η_p^2=0.224, $P<0.01$), $SkBF_{fing}$ ($F_{2.8, 53.3}$=4.9, η_p^2=0.204, $P<0.01$), CVC_{fing} ($F_{2.8, 53.1}$=4.2, η_p^2=0.182, $P<0.05$), and *MAP* ($F_{2.5, 48.1}$=3.3, η_p^2=0.148, $P<0.05$). $SkBF_{fing}$ in BR condition was significantly higher than in the water condition at 45- and 50-min rewarming ($P<0.05$). CVC_{fing} in BR condition was significantly higher than in water condition at 40- to 50-min rewarming ($P<0.05$).

Time course of $\bar{T}_{sk}$, T_{hand} and $T_{pro\text{-}dis}$ on non-immersed body region are shown in **Fig. 2**. During the rewarming phase, a significant main effect of time was detected in $\bar{T}_{sk}$ ($F_{2.1, 40.1}$=27.6, η_p^2=0.592, P<0.001) and $T_{pro\text{-}dis}$ ($F_{1.5, 28.6}$=3.7, η_p^2=0.163, P<0.05). A significant main effect of condition was detected in $T_{pro\text{-}dis}$ ($F_{1, 19}$=5.8, η_p^2=0.235, P<0.05). $T_{pro\text{-}dis}$ in BR condition was significantly smaller than the water condition at 30- to 50-min rewarming (P<0.05). No difference in T_{subl} between water and BR conditions (36.52±0.33 and 36.60±0.35°C) was observed at the end of rewarming phase.

The rewarming speeds in T_{fing} from 1 min to 5, 10, 15, and 20 min after hand immersion are presented in **Table 2**. Significantly faster rewarming was observed in beet condition compared to water at 15- and 20-min rewarming (both P<0.05). Similarly, the recovery speed in $SkBF_{fing}$ was significantly faster in BR condition at 15- and 20-min rewarming (both P<0.05).

The individual values of T_{fing} rewarming speeds in water and BR conditions are plotted in **Fig. 5**. Significant correlations were evident between conditions in 10, 15, and 20 min rewarming (r=0.69, P<0.01; r=0.68, P<0.01; r=0.58, P<0.01; respectively). The slope of the regression lines (95% CI) are 0.60 (0.28-0.91), 0.57 (0.27-0.88), and 0.43 (0.13-0.73) at 10, 15, and 20 min, respectively. The slopes of the regression lines (and 95% CI) were gentler compared to the y = x (reference line), which represents identical rewarming speed in water and BR conditions. Thus, the individuals with slower rewarming speed with water showed greater improvement in rewarming with BR supplementation.

Time course of thermal sensation of the whole-body and immersed hand, thermal comfort and pain sensation of the immersed hand are shown in **Fig. 4**. No difference in these subjective sensations was observed between conditions during the rewarming phase.

Discussion

This study investigated the effect of BR ingestion on CIVD response and rewarming of T_{fing} during and after hand immersion in cold water, by comparing with drinking water as a control. In contrast to our hypothesis for the immersion phase of the experiment, CIVD in T_{fing} and skin blood flow was not affected by BR. On the other hand, as a major finding of this study, rewarming of T_{fing} and skin blood flow was accelerated by drinking BR 2-hours before hand immersion. Hence, the hypothesis is only partially supported.

Cold-induced vasodilation

We originally hypothesised that CIVD would be enhanced by ingestion of BR, since it contains vasoactive ingredients such as NO_3^-, betanin, and chlorogenic acid (Wootton-Beard et al. 2011). However, the results showed no significant difference in all parameters of CIVD for T_{fing} and $SkBF_{fing}$ between BR and water

conditions during immersion. Additionally, thermal sensation and pain sensation of the immersed hand was not different between conditions.

Starting from the original observation of the hunting reaction of finger temperature to cold (Lewis 1930), the CIVD response has been studied in humans (Daanen and Ducharme 1999) and animals including adrenergic neural mechanism in isolated vascular smooth muscle (Rusch et al. 1981). Based on the current knowledge of the CIVD, NO-dependent active vasodilation and/or sympathetic withdrawal has been suggested as potential mechanisms of CIVD response (Daanen 2003; Cheung 2015). In this study, considering the significantly smaller $T_{pro\text{-}dis}$ in BR condition during the latter half of the hand immersion, whole-body vasodilation appears to be enhanced with BR supplementation during a prolonged (compared to other studies; e.g., Eglin et al., 2017; Hope et al., 2014) cold stimulus of the extremity. However, finger skin temperature and blood flow in the immersed hand did not show any effect of BR supplementation. This result was in line with the previous finding of no difference in CIVD response during hand immersion to 8°C water between BR and NO_3^--depleted placebo drink (Wickham et al. 2021). NO-mediated active vasodilation probably has a minor contribution to enabling the CIVD response when cold stimulus from the extremities is significant whilst also coupled with the effect of hydrostatic squeeze on the surface blood vessels of the immersed hand in healthy individuals. In persons with cold injury such as Raynaud's syndrome or those who are cold-sensitive with an abnormal endothelial function, NO supplementation is more likely to influence the vasomotor responses during cold challenge but not by a large magnitude (e.g., Shepherd et al. 2019; Hope et al. 2014). Hope et al. (2014) suggested that GTN, acting experimentally as the NO donor, bypasses the endothelium dependent NO pathway to re-establish the vascular response in cold-sensitive individuals. Hope et al. (2014) only observed facilitated post-immersion re-warming in their cold-sensitive group but not in their healthy controls. Shepherd et al. (2019) showed some transient evidence of increased CVC with chronic supplementation with BR and nitrate depleted BR but not acute supplementation indicating a greater dose of BR than used here might be needed to induce CIVD during cold immersion. Collectively, there is now a growing body of evidence that suggests acute BR supplementation doesn't have the potency to evoke vasodilation during local cold challenge in healthy (Wickham et al. 2021) and cold-sensitive/injured individuals (Eglin et al. 2013; Shepherd et al. 2019). Thus, it is speculated that release of vasoconstrictor tone might be one of the major mechanisms for the CIVD response rather than NO-dependent active vasodilation. On the other hand, systemic effects of BR supplementation on blood pressure (Shepherd et al. 2019; Wickham et al. 2021) are plausible, as shown in the observation of higher distal skin temperature during prolonged hand immersion to this body of evidence (see Fig. 2). Therefore, BR could also evoke vasodilatory effects in nonglaborous regions where noradrenergic vasoconstrictor nerves and cholinergic active vasodilator nerves are active (Kellogg 2006). Nevertheless, our observations of accelerated rewarming following cold exposure are novel and require further exploration.

346
347 ***Acceleration of rewarming***
348 The major finding of this study was that BR ingestion accelerated the rewarming speed of T_{fing} and finger
349 skin blood flow after 30-min hand cold immersion. During the rewarming phase, after terminating the cold
350 stimulus, the vasoactive ingredients in BR enhanced the whole-body and local vasodilation but only after
351 10 ($SkBF_{fing}$) to 15 minutes (T_{fing}) of rewarming (Table 2). The duration of rewarming studied here might
352 also be another reason why prior studies have not revealed this difference having primarily measured for
353 up to 10-min of rewarming (Eglin et al. 2017; Shepherd et al. 2019). It is evident from the present data that
354 the experimental effects of BR probably extend beyond the 20-min rewarming period where these
355 significant differences remained. Although rewarming speed in $SkBF_{fing}$ and T_{fing} was accelerated with BR,
356 it was not associated with perception of local thermal sensation or whole-body thermal comfort.
357
358 One of the most potent vasoactive ingredients in BR is NO_3^-, with many studies reporting NO_3^- - NO_2^- -NO
359 pathway induced vasodilation and consequently lowered blood pressure through exogenous NO mediated
360 relaxation of endothelial cells to relax vascular smooth muscle (Wylie et al. 2013; Lara et al. 2016; Richards
361 et al. 2018). Unlike the present study, previous studies reported no effect of acute BR ingestion on
362 peripheral vasodilatory response following 2-min cold immersion of extremities in comparison with NO_3^--
363 depleted placebo (Eglin et al. 2017; Shepherd et al. 2019). The short duration (2 min) cold immersion in
364 the previous studies and prolonged (30 min) cold exposure in the present study are categorized into early-
365 phase (i.e., primarily skin cooling) and late-phase local cooling (i.e., skin and superficial muscle cooling),
366 respectively (Hodges and Johnson 2009; Alba et al. 2019). At the onset of the local cold stimulus, early
367 vasoconstriction is induced mostly via adrenergic and neural mechanisms (Ekenvall et al. 1988; Stephens
368 et al. 2004), whereas later vasoconstriction during prolonged cooling is mediated via combination of
369 continued vasoconstrictor nerve excitation and inhibition of NO vasodilator pathway (Hodges et al. 2006;
370 Hodges and Johnson 2009; Alba et al. 2019). It is likely that there is a more substantial reduction in
371 bioavailable NO at the end of 30-min hand immersion in the present study due to the decreased activity of
372 endothelial NOS and downstream of NOS (Hodges et al. 2006) and hence this is the reason for the efficacy
373 of increasing NO in the present study. The likely reduction of NO bioavailability during longer cold
374 exposure enabled us to find a significant vasodilatory effect of BR, as external NO donor via NO_3^- - NO_2^- -
375 NO pathway, accelerating T_{fing} rewarming following the cold exposure. Yet, the time course of our $SkBF_{fing}$
376 and T_{fing} (**Table 2**) data during rewarming along with the magnitude of T_{fing} change (**Fig 3**) suggest plausible
377 successive relief to both mechanisms (i.e., relief of vasoconstrictor nerve excitation and restoration of the
378 NO vasodilator pathway; Hodges et al. 2006; Hodges and Johnson 2009; Alba et al. 2019) that are
379 associated with prolonged cooling. $SkBF_{fing}$ laser doppler flowmetry data from Hodges et al. (2018) during
380 30-min hand immersion indicate the onset of the CIVD response precedes increases in T_{fing} (i.e., the same
381 as our data) but closely matched the measured neurogenic activity in the finger but not the endothelial nitric

382 oxide dependent or independent activity (Hodges et al. 2018). The changes seen in T_{fing} in the study of
383 Hodges et al. (2018) were small and transient due to the ongoing cold-water immersion. During rewarming
384 in the present study, we suggest the BR supplementation facilitated the earlier onset of vasoconstrictor
385 nerve relief, facilitating an increase in $SkBF_{fing}$ at 10-min of rewarming and accelerating the increase in T_{fing}
386 from 15-minutes. This increase in $SkBF_{fing}$ raises T_{fing} towards the vasomotor range (i.e., 26°C, (Folkow
387 and Neil 1971); see **Fig 3**) where further active vasodilation is plausible (Kellogg et al. 1998; Kellogg
388 2006).
389
390 The initial reduction of NO bioavailability, including the inhibition of NOS activity, is partly induced by
391 the elevation of reactive oxygen species (ROS) from vascular smooth muscle mitochondria (Bailey et al.
392 2005; Holowatz and Kenney 2007; Hodges and Johnson 2009; Johnson et al. 2014). In addition, the ROS
393 generated by local cooling also enhances Rho-kinase activity to increase vascular tone to the neural
394 noradrenalin release, which is mainly explained by the translocation of α_{2c}-adrenoreceptors to the surface
395 of vascular smooth muscle cell (Bailey et al. 2004; Bailey et al. 2005; Hodges and Johnson 2009). The Rho-
396 kinase pathway is relatively slow event as shown in time course of Rho activity to cooling in human cultured
397 dermal arteriolar vascular smooth muscle (Bailey et al. 2004). Thus, the enhanced vasodilatory response
398 with BR ingestion after prolonged cold exposure might also be related to slow reversal of the ROS and
399 Rho-kinase pathway which fits with the time course of responses shown in the present study. Previous
400 research investigating the vasomotor effect of BR supplementation suggested the nitrate-independent
401 vasodilatory response due to other bioactive ingredients in BR (Bahadoran et al. 2017; Thompson et al.
402 2018; Shepherd et al. 2019). Among them betanin was focused on as one of the phytochemical antioxidants
403 in BR (Esatbeyoglu et al. 2015; Hadipour et al. 2020). A study reported that local administration of
404 ascorbate antioxidant inhibited the vasoconstriction during local skin cooling (Yamazaki 2010). Thus, it
405 was suggested that quenching of ROS by the antioxidants might decrease efficacy of adrenoreceptors and
406 influence the vascular response to cooling. The antioxidant, betanin contained in BR, inhibits the diffusion-
407 controlled reaction of NO with superoxide (i.e., an ROS) by scavenging super-oxide radicals that create
408 peroxynitrite thereby slowing the appearance of this ROS and improving the bio-availability of NO
409 (Sakihama et al. 2012). Hence, consumption of betanin is a plausible means to increase bioavailable NO
410 for stimulating vasodilation (Esatbeyoglu et al. 2015). In the present study, 30-min hand immersion in 8°C
411 was probably sufficient oxidative stress to decrease bioavailable NO (Christmas et al. 2016). Following this
412 cold stress, the combined effect of nitrate (NO production) and betanin (antioxidation) in BR could increase
413 bioavailable vasodilatory NO, which could plausibly enhance T_{fing} rewarming compared to the water
414 ingestion condition following initial relief to vasoconstrictor nerve activity. It is a limitation of the present
415 study that we cannot discern the separate contribution of the bioactive ingredients (e.g., nitrate and betanin)
416 in BR that contribute to the responses we report and that we could not blind the treatment conditions;

417 although it did not evoke significant change in thermal sensation and comfort, both scenarios require further
418 research. Lastly, our observations are restricted to males only.
419
420 ***Individual variation in the effect of beetroot supplementation***
421 There are individual variations in vasomotor response to local cooling. Cold sensitive individuals such as
422 with non-freezing cold injury (NFCI) and Raynaud's syndrome present with colder hands and foot skin
423 temperature, greater vasoconstriction to cold and slower rewarming rate following cold exposure compared
424 to normal individuals (Eglin et al. 2013; O'Reilly et al. 1992). Patients with Raynaud's phenomenon show
425 deficiency of NO in response to cold stimulus (Tanaka et al. 2012), which could be due to their greater
426 oxidative damage resulting from higher serum ROS level (Biondi et al. 2008). It was also reported that
427 increased ROS in response to cooling might be one of the mediators for tissue damage in NFCI (Geng et
428 al. 2015). Moreover, even in healthy people with cold constitution ("hi-e-sho" in Japanese), greater
429 vasoconstriction was provoked to local skin cooling and iontophoretic noradrenaline application (Yamazaki
430 2015). These findings indicated that cold sensitive individuals would have an increased sensitivity of
431 adrenoreceptor on vascular smooth muscle and/or decreased bioavailability of NO, that has been shown to
432 evoke oxidative stress (Biondi et al. 2008).
433
434 In the present study, individual variation in healthy participants was observed in the rewarming speed after
435 30-min cold exposure. The slopes of the regression lines between the rewarming speed in water and BR
436 conditions were gentler compared to the y = x, which represents identical speed in both conditions. This
437 result indicated that cold sensitive individuals with slower rewarming in the water condition showed more
438 remarkable improvement in the rewarming speed with BR ingestion. We speculate that cold sensitive
439 individuals, who potentially have more oxidative stress and less bioavailable NO, could benefit more so
440 following BR supplementation containing antioxidants like betanin and NO_3^- as NO donor; a focus of future
441 research.
442
443 **Conclusions**
444 This study investigated the effect of beetroot supplementation on vasomotor responses during and after
445 hand cold immersion in human. CIVD in finger temperature and skin blood flow was not affected by BR
446 ingestion, but a significantly smaller $T_{pro\text{-}dis}$ in BR condition, primarily due to higher distal temperature,
447 would indicate that whole-body vasodilation was enhanced during the local cold stimulus. The major
448 finding of this study was that BR accelerated rewarming in finger skin temperature and skin blood flow
449 after the local cold stimulus. Additionally, cold sensitive individuals with a slower rewarming rate with
450 water demonstrated more remarkable acceleration in rewarming with BR supplementation.
451
452 **Declarations**

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Conflicts of interest None declared.

Ethics approval All experimental protocols in this study were approved by the IRB of Hokkaido University.

Author contributions HW and MB conceived and designed research. KS and SS conducted the experiments. KS and HW analyzed data. HW and MB wrote the draft of manuscript. All authors read and approved the manuscript.

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