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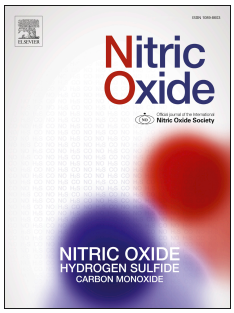


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# Dietary nitrate supplementation increases nitrate and nitrite concentrations in human skin interstitial fluid

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**Author's contributions**:

N.F. conceived and designed experiments. N.F., S.O., Y.K., and G.M., contributed to data collection and analysis. N.F., S.O., Y.K., G.M., S.J.B., A.B.L., J.T.A., T.A., Y.T., N.O., K.W., and T.N. interpreted the experimental results. N.F. drafted the manuscript. All authors edited and revised the manuscript and approved the final version of the manuscript. All experiments took place at the Faculty of Health and Sport Sciences, University of Tsukuba, Tsukuba, Japan.

**Data availability:**

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Declarations of interest**:

None

**ABSTRACT (242/250 WORDS)**

Acute dietary nitrate ($NO_3^-$) supplementation can increase [$NO_3^-$], but not nitrite ([$NO_2^-$]), in human skeletal muscle, though its effect on [$NO_3^-$] and [$NO_2^-$] in skin remains unknown. In an independent group design, 11 young adults ingested 140 mL of $NO_3^-$-rich beetroot juice (BR; 9.6 mmol $NO_3^-$), and 6 young adults ingested 140 mL of a $NO_3^-$-depleted placebo (PL). Skin dialysate, acquired through intradermal microdialysis, and venous blood samples were collected at baseline and every hour post-ingestion up to 4 h to assess dialysate and plasma [$NO_3^-$] and [$NO_2^-$]. The relative recovery rate of $NO_3^-$ and $NO_2^-$ through the microdialysis probe (73.1% and 62.8%), determined in a separate experiment, was used to estimate skin interstitial [$NO_3^-$] and [$NO_2^-$]. Baseline [$NO_3^-$] was lower, whereas baseline [$NO_2^-$] was higher in the skin interstitial fluid relative to plasma (both $P<0.001$). Acute BR ingestion increased [$NO_3^-$] and [$NO_2^-$] in the skin interstitial fluid and plasma (all $P<0.001$), with the magnitude being smaller in the skin interstitial fluid (e.g., 183±54 vs. 491±62 μM for $\triangle$[$NO_3^-$] from baseline and 155±190 vs. 217±204 nM for $\triangle$[$NO_2^-$] from baseline at 3 h post BR ingestion, both $P\leq0.037$). However, due to the aforementioned baseline differences, skin interstitial fluid [$NO_2^-$] post BR ingestion was higher, whereas [$NO_3^-$] was lower relative to plasma (all $P<0.001$). These findings extend our understanding of $NO_3^-$ and $NO_2^-$ distribution at rest and indicate that acute BR supplementation increases [$NO_3^-$] and [$NO_2^-$] in human skin interstitial fluid.

**Key words:**

Interstitium; extracellular space; nitric oxide; reduction; intradermal microdialysis

## 1. INTRODUCTION

Increased dietary nitrate ($NO_3^-$) intake, which can be achieved through greater consumption of $NO_3^-$-rich foods such as beetroot juice (BR) and green leafy vegetables, can improve various physiological responses [1; 2; 3] and aspects of exercise performance [4; 5]. These positive effects of dietary $NO_3^-$ supplementation are attributed to increased nitric oxide (NO) availability delivered through the $NO_3^-$-$NO_2^-$-NO pathway. It is well documented that, blood [$NO_3^-$] and [$NO_2^-$] increase following consumption of $NO_3^-$-rich foods [6; 7]. Recent studies have also reported that chronic dietary $NO_3^-$ supplementation lasting 5 days or more can increase [$NO_3^-$] in the skeletal muscle and liver in rats [8; 9]. In human studies, a single dose of $NO_3^-$supplementation acutely increases both salivary [$NO_3^-$] and [$NO_2^-$] [7], and increases [$NO_3^-$], but not [$NO_2^-$], in skeletal muscle and urine [7; 10; 11; 12]. Moreover, diet-induced increases in skeletal muscle [$NO_3^-$] appear to be functionally important during $NO_3^-$ deprivation or exercise [10]. However, it remains to be determined if dietary $NO_3^-$ supplementation increases [$NO_3^-$] and/or [$NO_2^-$] in human skin wherein NO is thought to play a role in modulating several functions including skin vascular tone [13], sweating [14; 15], barrier function [16], wound heating [17], and melanin synthesis [18].

Skin is a very large organ with a total area of 1.5-2.0 $m^2$ in adults. Skin also possesses an interstitium, which comprises a contiguous and widespread fluid filled space that operates as the interface between the systemic circulation and dermis cells [19]. Thus, the skin interstitium might have a capacity to store nutrients delivered exogenously through the diet. Indeed, a recent study reported that skin can store sodium [20]. It has also been reported that skin interstitial fluid glucose and caffeine concentrations are increased following oral glucose and caffeine ingestion [21]. The molecular mass of $NO_3^-$

and $NO_2^-$ is 62 and 46 g/mol, respectively both of which are lower than that of glucose (180 g/mol) and caffeine (194 g/mol). Thus, it seems plausible that $NO_3^-$ and $NO_2^-$ as relatively small molecules might pass capillary walls in a similar manner to glucose and caffeine. If so, $NO_3^-$ rich food has the potential to acutely increase [$NO_3^-$] and [$NO_2^-$] in skin interstitial fluid, though this hypothesis has yet to be directly investigated.

Baseline [$NO_3^-$] and [$NO_2^-$] in human skin epidermis and dermis are greater relative to plasma [23], with most $NO_3^-$ and $NO_2^-$ found in the epidermis [24]. Therefore, in addition to endogenous increases in [$NO_3^-$] and [$NO_2^-$], via oxidation of NO and $NO_2^-$, it is possible that exogenous diet-induced increases in [$NO_3^-$] and [$NO_2^-$] in the skin interstitial fluid could subsequently migrate to the epidermis to be stored as $NO_3^-$ and $NO_2^-$, in accord with responses observed in the human skeletal muscle tissue [7; 10; 11; 12]. Given that baseline human muscle interstitial [NOx], which reflects a combination of [$NO_3^-$] and [$NO_2^-$], was nearly double that in venous plasma [25], interstitial fluid may also have a capacity to store $NO_3^-$ and $NO_2^-$. However, it has yet to be determined whether baseline [$NO_3^-$] and [$NO_2^-$] are higher and increased to a greater extent after $NO_3^-$ rich BR ingestion in skin interstitial fluid compared to plasma.

The purpose of the present study was to test the hypotheses that baseline [$NO_3^-$] and [$NO_2^-$] would be higher and [$NO_3^-$] and [$NO_2^-$] would increase to a greater extent after $NO_3^-$ rich BR ingestion, in human skin interstitial fluid vs. venous plasma.

## 2. MATERIALS AND METHODS

### 2.1 Ethical approval

The current study was approved by the Human Subjects Committee of the University of Tsukuba (#29-24), adhering to the latest version of Declaration of Helsinki. Verbal and

written informed consent were obtained from all participants before commencing the experiment.

**2.2 Participants**

Nineteen participants were divided into BR (11 adults including 4 females) and placebo (PL, 8 adults including 2 females) groups. However, due to vasovagal reflex occurring in two of the eight participants in the PL group during blood sampling, the total number of PL group was six (6 adults including 2 females). Since no relevant previous data is available, a sample size calculation was not performed for the present study. Age, body mass, height, and body mass index (mean ± standard deviation) were, respectively, 23 ± 3 years, 61.3 ± 7.6 kg, 1.68 ± 0.04 m, and 21.7 ± 2.1 kg/m$^2$ for the BR group, and 22 ± 1 years, 67.4 ± 8.8 kg, 1.66 ± 0.04 m, and 24.6 ± 2.6 kg/m$^2$ for the PL group. All participants were non-smokers, otherwise healthy individuals, without any history of chronic disease and therefore not on any prescription medication. No attempt was made to control for female menstrual cycle as there is no study demonstrating the influence of menstrual phase on skin interstitial [$NO_3^-$] and [$NO_2^-$] kinetics. Participants were asked not to consume alcohol or caffeine, and to avoid any strenuous exercise for up to 12 h prior to the experiment, and not to consume any food for up to 2 h prior to the start of the experiment. Given that oral bacteria have been shown to reduce $NO_3^-$ to $NO_2^-$ [26], participants were instructed to refrain from utilizing antibacterial toothpaste and mouthwash for the duration of the study. Moreover, they were instructed to avoid consumption of chewing gum and confectionaries that contained antibacterial substances such as chlorhexidine and xylitol over the same time period.

### 2.3 Experimental protocol

A schematic of the experimental protocol is illustrated in Figure 1. Upon arrival, participants were asked to sit, where they remained resting at an environmental temperature of ~25 ºC for the duration of the trial. An intradermal microdialysis fiber, consisting of a commercially available 10 mm regenerated cellulose membrane with a 50 kDa cutoff (EICOM, Kyoto, Japan), was then placed into the dermis of left forearm skin. To place the fiber, a 25-G needle was introduced to the forearm skin. The fiber was then advanced along the needle. The needle was then withdrawn leaving the fiber in the skin (~1 mm in depth). The fiber was continuously perfused with lactated Ringer's solution (Fuso Pharmaceutical Industries, Osaka, Japan) at a rate of 2 μL/min by a microinfusion pump (BASi Bee Hive controller and Baby Bee syringe drive; Bioanalytical Systems, West Lafayette, IN, USA). Following a minimum 60 min phase of lactated Ringer's perfusion, the duration of which is sufficient for the trauma associate with fiber insertion to subside [27], a 15 min of baseline dialysate sample for assessment of interstitial [$NO_3^-$] and [$NO_2^-$] was taken. At the termination of the baseline measurement, participants then ingested either 140 mL of BR (Beet-It Stamina Shot, James White drinks Ltd. Ipswich, UK) containing 9.68 mmol $NO_3^-$ (0.595 g of $NO_3^-$) or 140 mL of an equivalent $NO_3^-$ depleted PL, identical in taste, color, and smell (James White drinks Ltd). Venous blood samples were collected from median antebrachial, cephalic, or basilic veins at 0 h (pre-consumption value), and at 1, 2, 3, and 4 h post-consumption of the supplements. Venous blood sampling from one male participant in the BR group was unsuccessful (n = 10). Skin dialysate was collected throughout the 15 min of baseline period prior to supplement ingestion, and during the final 15 min of each hour, up to 4 h, post-consumption period

(15 min × 2 μL/min = 30 μL). Skin temperature across an area of 8.03cm$^2$ at the intradermal skin site was clamped at 33 ºC by a local heating unit (Ø 32 mm, PeriFlux heater PF 450) covering the intradermal microdialysis fiber. Blood pressure was measured from the right brachial artery using an automated sphygmomanometer (TM-2580, A&D Company, Limited, Tokyo, Japan) twice at each stage (baseline, 1, 2, 3, and 4 h post supplement consumption), from which mean arterial pressure was determined.

**2.4 Assessment of dialysate and venous plasma [$NO_3^-$] and [$NO_2^-$]**

To eliminate potential contamination of $NO_3^-$ and $NO_2^-$, all sample tubes, spatulas, and glassware were thoroughly rinsed with deionized water prior to initiation of the experiment and analysis of [$NO_3^-$] and [$NO_2^-$] in venous plasma and skin dialysate samples. Venous blood samples of ~5 mL were collected into EDTA vacutainers (Nipro, Osaka, Japan). Once collected, blood samples were immediately centrifuged at 1,350 x g at 4 ºC for 10 min to obtain a plasma sample. Skin dialysate of ~30 μL (15 min × 2 μL/min) was collected for 15 min into a 0.65 mL of microtube (#11150, Sorenson Bioscience Inc., Salt Lake City, USA) at baseline and each post-consumption timepoint. Perfusion through the microanalytical fibers was continuously monitored by the investigators at intervals of 5-10 min. Both the plasma and skin dialysate samples were frozen at –80 ºC for storage until they were analyzed. [$NO_3^-$] and [$NO_2^-$] were assessed in duplicate using Griess reaction by injecting 10 μL sample to ion chromatography (Eicom ENO-20 $NO_x$– analysis system, Eicom, Kyoto, Japan), as was done in previous studies wherein the effect of BR ingestion on venous plasma [$NO_3^-$] and [$NO_2^-$] was assessed [28; 29]. In our pilot work, venous plasma [$NO_3^-$] or [$NO_2^-$] was comparable between 10 μL vs. 20 μL sample injections, validating the use of a 10 μL sample. [$NO_3^-$] and [$NO_2^-$] was

determined as the mean of duplicate measurements for both dialysate and venous plasma. Immediately prior to analysis, plasma samples were mixed with an equal volume of methanol (Nacalai Tesque, Kyoto, Japan) and subsequently was centrifuged at 1,350 x g at 4 ºC for 10 min for deproteinization. Between-sample coefficient of variation for the plasma [$NO_3^-$] and [$NO_2^-$] and skin dialysate [$NO_3^-$] and [$NO_2^-$] were 8.28%, 2.32%, 3.65%, and 3.84%, respectively.

**2.5 Pre-experimental work to assess fiber recovery rate**

[$NO_3^-$] and [$NO_2^-$] measured in dialysate would be expected to be lower than those in the skin interstitial fluid, as migration of $NO_3^-$ and $NO_2^-$ from the skin interstitial fluid to dialysate is limited within a short period. To better understand this issue, on a separate occasion, we conducted an experiment to specifically estimate recovery rate of $NO_3^-$ and $NO_2^-$ through microdialysis, after which we could estimate skin interstitial [$NO_3^-$] and [$NO_2^-$]. In accordance with the procedures established in the main experiment, all sample tubes, spatulas, and glassware were meticulously cleaned with deionized water before commencing the experiment and assessing [$NO_3^-$] and [$NO_2^-$] in the pre-experimental work to mitigate the possibility of $NO_3^-$ and $NO_2^-$ contamination. In this study, three microdialysis membranes were immersed into a beaker containing 50 mL of lactated Ringers solution (Fuso Pharmaceutical Industries). The three microdialysis membranes were continuously perfused with lactated Ringers solution at a rate of 2.0 μL/min. The baseline measurement was conducted for 45 min. Thereafter, the three membranes were transferred into another beaker containing 1000 nmol [$NO_3^-$] and [$NO_2^-$] dissolved in 50 ml of lactated Ringers solution and remained for 45 min. Beaker dialysate of ~60 μL (30 min × 2 μL/min) was collected during the last 30 min of each stage (baseline and 1000

nmol standard perfusion periods). We also collected ~60 μL of lactated Ringer solution from each beaker at the beginning and end points of each stage. The temperature of solution in beakers was maintained at 37 °C throughout. $[NO_3^-]$ and $[NO_2^-]$ in the beaker dialysates and beakers were analyzed in duplicate as explained previously (*See section*, Assessment of dialysate and venous plasma $[NO_3^-]$ and $[NO_2^-]$). Mean $[NO_3^-]$ and $[NO_2^-]$ concentrations in the beakers were estimated as the mean of the two values obtained at the beginning and end points of each stage. Between-sample coefficient of variation for the beaker dialysate $[NO_3^-]$ and $[NO_2^-]$ and beaker $[NO_3^-]$ and $[NO_2^-]$ were 5.31%, 10.57%, 5.53%, and 15.26%, respectively. Relative recovery rate was calculated for $NO_3^-$ and $NO_2^-$ using the equation below:

$$\text{Relative recovery (\%)} = (C_{\text{beaker dialysate}}/C_{\text{beaker}}) \times 100$$

Where $C_{\text{beaker dialysate}}$ is the concentration of beaker dialysate collected during standard perfusion period minus the concentration obtained during baseline; $C_{\text{beaker}}$ is the mean concentration of beaker during standard perfusion period minus the concentration obtained during baseline. Relative recovery was obtained by averaging data over the three microdialysis membranes.

Using the relative recovery calculated, $[NO_3^-]$ and $[NO_2^-]$ in the skin interstitial fluid ($C_{\text{skin interstitial fluid}}$) was estimated using the equation below:

$$C_{\text{skin interstitial fluid}} = C_{\text{skin dialysate}} \times [100 / \text{relative recovery (\%)}]$$

Where $C_{skin\ dialysate}$ is the concentration of skin dialysate collected during the BR ingestion experiment.

**2.6 Data analyses**

Delta (△) change in $[NO_3^-]$ or $[NO_2^-]$ from baseline was calculated for each sample (i.e., skin interstitial fluid and plasma). Area under the curve of $[NO_3^-]$ or $[NO_2^-]$ over the course of 4-h post-consumption period was also assessed. To compare baseline $[NO_3^-]$ or $[NO_2^-]$ between the skin interstitial fluid and plasma, data collected from the BR and PL groups were pooled (n = 16). All values are reported as means ± standard deviation.

**2.7 Statistical analyses**

Inferential statistical analysis was conducted using Prism 9.4.0 (GraphPad, CA, USA). Alpha was set a-priori at 0.05. Normal distribution of data was verified by Q-Q plot. Baseline $[NO_3^-]$ or $[NO_2^-]$ was compared between the skin interstitial fluid and plasma using the two-tailed paired t-test. $\triangle[NO_3^-]$ and $\triangle[NO_2^-]$ from their respective baseline were compared between the skin interstitial fluid and plasma using the two-tailed paired t-test at each post-consumption period. A two-way repeated measure ANOVA was used for $[NO_3^-]$ or $[NO_2^-]$ with two factors of time (baseline, 1, 2, 3, and 4 h into post-consumption) and sample (skin interstitial fluid and plasma). When an interaction between time and sample was detected, the skin interstitial fluid and plasma were compared at each post-consumption period with Bonferroni's multiple comparison test. Blood pressures were analyzed using a one-way repeated measure ANOVA with a factor of time, followed by Bonferroni's multiple comparison test. Correlation analysis between the skin interstitial fluid and plasma for $[NO_3^-]$ or $[NO_2^-]$ was conducted using a two-

tailed parametric Pearson product-moment correlation coefficient.

## 3. RESULTS

Relative recovery of the microdialysis probe was 73.1% for $NO_3^-$ and 62.8% for $NO_2^-$, allowing subsequent skin interstitial fluid [$NO_3^-$] and [$NO_2^-$] to be estimated from sampled dialysate.

Baseline [$NO_3^-$] was ~2 time lower, whereas baseline [$NO_2^-$] was ~5 times higher in the skin interstitial fluid vs. venous plasma (17±19 vs. 31±25 μM for [$NO_3^-$] and 269 ±77 vs. 55±48 nM for [$NO_2^-$], both $P < 0.001$, Figure 2). Drinking BR increased [$NO_3^-$] and [$NO_2^-$] in both the skin interstitial fluid and venous plasma (all $P < 0.001$, Figure 3A, D). The increases in [$NO_3^-$] were 2.6-4.2 times greater in the venous plasma vs. skin interstitial fluid (e.g., 183±54 vs. 491±62 μM at 3 h post-consumption, all $P < 0.001$, Figure 3B), and the increases in [$NO_2^-$] were 1.3-1.7 times greater in the venous plasma relative to skin interstitial fluid at 2 h and 3 h post-consumption (e.g., 155±190 vs. 217 ±204 nM for [$NO_2^-$] at 3 h post-consumption, $P = 0.037$, Figure 3E). Similar results were also observed when area under the curve between post-consumption period (h) and [$NO_3^-$] or [$NO_2^-$] was assessed (both $P \leq 0.025$, Figure 3C, F). However, given that baseline [$NO_2^-$] in the skin interstitial fluid was much greater than the plasma as noted above, [$NO_2^-$] was greater in the skin interstitial fluid vs. venous plasma throughout post-consumption periods (Figure 3D, $P < 0.001$).

Increases in [$NO_3^-$] in the skin interstitial fluid were not correlated with those in the plasma at each post-consumption period (all $P \geq 0.132$, Figure S1A-D), but significant correlations were observed when data collected from 1-4 h post-consumption periods were pooled ($P = 0.024$, Figure 4A). Increases in the [$NO_2^-$] in the skin interstitial fluid

were correlated with those in the plasma at 2, 3, and 4 h post-consumption as well as when data collected throughout the post-consumption periods were pooled (all $P \leq 0.009$, Figure 4B, Figure S1F-H).

Blood pressures remained unchanged throughout the post-BR consumption period relative to the pre-drink values (Figure 5A-C, all $P \geq 0.678$). The effect of BR on skin blood flow indices was reported in a supplemental file (Figure S2-3 and associated text). PL had no clear effects on all variables assessed (Figure S4-5).

## 4. DISCUSSION

### 4.1 $[NO_3^-]$ and $[NO_2^-]$ in the skin interstitial fluid

Pooled data from BR and PL groups showed that, in contrast to our hypothesis, baseline $[NO_3^-]$ was ~2 fold lower in skin interstitial fluid relative to venous plasma. Lower baseline $[NO_3^-]$ in the skin interstitial fluid might reflect that $NO_3^-$ in the skin interstitial fluid rapidly moves to intracellular space of skin tissues leading to $NO_3^-$ storage in the skin as has been observed in human skeletal muscle [7; 10; 11; 12]. Indeed, it has been previously reported that human skin epidermis exhibited greater $[NO_3^-]$ relative to plasma [24]. However, while the $NO_3^-$ transporters, sialin and chloride channel 1, exist in skeletal muscle [11; 30], and salivary glands possess sialin [31], a direct assessment is required to elucidate if sialin and/or chloride channel 1 is/are expressed in human skin epidermis. In contrast to $[NO_3^-]$, and consistent with our hypothesis, baseline $[NO_2^-]$ was ~ 5-fold higher in skin interstitial fluid than in venous plasma. Although $NO_2^-$ can be accumulated in the heart tissue via phospholipid membrane in the form of undissociated nitrous acid ($HNO_2$) [32] and in the erythrocytes through anion exchange protein [33], $NO_2^-$ might not be transported into intracellular space in the skin epidermis, underlying the very high $[NO_2^-]$

in the skin interstitial fluid. If this is true, the reported higher [$NO_2^-$] in the human skin epidermis [24] might be mainly due to reduction of [$NO_3^-$] to [$NO_2^-$], presumably through xanthine oxidoreductase [10], though this possibility needs a direct evaluation in future.

In the present study, ingestion of $NO_3^-$ rich BR increased [$NO_3^-$] and [$NO_2^-$] in venous plasma, a response consistent with several previous human studies [6; 7; 34]. We extended these previous findings by demonstrating that BR ingestion also increased [$NO_3^-$] and [$NO_2^-$] in skin interstitial fluid in accordance with our hypothesis. As small molecules are capable of passively diffusing through skin capillary endothelial cells [22], this likely accounted for increased [$NO_3^-$] and/or [$NO_2^-$] in the intravascular space after BR ingestion subsequently accumulating in the extravascular space, thereby increasing skin interstitial fluid [$NO_3^-$] and [$NO_2^-$]. This is indirectly supported by our observation that [$NO_3^-$] or [$NO_2^-$] in the skin interstitial fluid and plasma were positively correlated.

BR ingestion increased both [$NO_3^-$] and [$NO_2^-$] in skin interstitial fluid and venous plasma from baseline with the increases in [$NO_3^-$] and [$NO_2^-$] being lower in skin interstitial fluid by ~300μM and ~50nM, respectively. However, since baseline [$NO_3^-$] and [$NO_2^-$] largely differed between skin interstitial fluid and venous plasma as discussed above, skin interstitial fluid [$NO_2^-$] remained higher, whereas skin interstitial fluid [$NO_3^-$] was lower than venous plasma after BR ingestion. The lower [$NO_3^-$] in skin interstitial fluid after BR ingestion could be associated with the movement of $NO_3^-$ from the interstitial fluid to intracellular space via sialin and/or chloride channel 1, but the existence of these $NO_3^-$ transporters needs to be directly confirmed in future as discussed above. In addition, ~25% of $NO_3^-$ in the blood appears to be absorbed in the salivary gland acinar cells through sialin [31], and ~60% is extracted by the kidneys [35], reducing the amount of $NO_3^-$ available to enter into the interstitial fluid from intravascular space. On

the other hand, $NO_3^-$ in the skin interstitial fluid might be reduced to $NO_2^-$ due to reductases such as xanthine oxidoreductase, contributing to the smaller increase in [$NO_3^-$] in the skin interstitial fluid. Indeed, xanthine oxidoreductase appears to be present and functionally active in the human skin, as indirectly evidenced by the local administration of allopurinol in previous work, a known xanthine oxidoreductase inhibitor, resulting in increased skin blood flow during local skin heating [36]. Indeed, the reduction of $NO_3^-$ to $NO_2^-$ in skin interstitial fluid is indirectly supported by our results showing that [$NO_2^-$] in the skin interstitial fluid was much higher than that in the plasma before and after ingestion of BR.

### 4.2 Physiological significance

The physiological significance of the elevated [$NO_3^-$] and [$NO_2^-$] in the skin's interstitial fluid resulting from ingestion of BR remains unknown, though the impact of BR ingestion on regulation of skin blood flow appears to be minimal (see Figure S2-3 and associated text). Since NO is involved in barrier function [16], wound heating [17], and melanin synthesis [18] in skin, increased $NO_3^-$ and $NO_2^-$ in the skin interstitial fluid might modulate these functions. The application of this knowledge might extend to clinical settings in which elevated skin interstitial [$NO_3^-$] and [$NO_2^-$] could be utilized as a clinical tool, for example, the use of exogenous $NO_3^-$ to pre-condition skin prior to free-flap surgery, to better ensure tissue survival [37]. Future studies are warranted to test these possibilities (Figure S6).

### 4.3 Considerations

This study has eight considerations to note. First, diet was not controlled in the current

study as we wanted the study to reflect free-living conditions in order to improve ecological validity. However, in doing so, we cannot exclude the possibility that diet prior to the experimental trials may have modulated our results. Second, blood and dialysate were only collected up to 4 h after ingesting BR and as such it is unclear how long [$NO_3^-$] and [$NO_2^-$] in the skin interstitial fluid are elevated above baseline values. Third, we assessed the acute effect of BR only. Hence, we do not know how chronic ingestion of BR modulates [$NO_3^-$] and [$NO_2^-$] in skin interstitial fluid. Forth, we did not control for female menstrual cycle, such that we do not know if the responses assessed in the present study differ depending on menstrual phase. Fifth, we assessed [$NO_3^-$] and [$NO_2^-$] in the skin interstitial fluid at the forearm only. It remains to be determined if similar responses are observed in non-forearm skin sites. Sixth, we did not assess specific mechanisms by which [$NO_3^-$] and [$NO_2^-$] increased in the skin interstitial fluid after ingestion of BR, which needs to be assessed in future. Seventh, a recent study demonstrated that BR ingestion increased circulating [S-nitrosothiols] [38], a reactive nitrogen intermediate that can function as a NO donor. We do not know if BR ingestion increases [S-nitrosothiols] in the skin interstitial fluid. Eighth, our study was not designed to assess physiological significance of the elevated [$NO_3^-$] and [$NO_2^-$] in the skin interstitial fluid, beyond blood pressure and skin perfusion, which needs to be assessed in future research.

## 5. Conclusion

Baseline [$NO_3^-$] was lower, whereas baseline [$NO_2^-$] was higher, in skin interstitial fluid than venous plasma. Acute BR supplementation was effective at increasing [$NO_3^-$] and [$NO_2^-$] in both skin interstitial fluid and venous plasma, but elevations in [$NO_3^-$] and [$NO_2^-$] above baseline values were lower in skin interstitial fluid by ~300μM and ~50nM,

respectively. However, despite smaller increases in [$NO_3^-$] and [$NO_2^-$] above baseline in skin interstitial fluid after BR ingestion, skin interstitial fluid [$NO_2^-$] remained higher than in venous plasma, whereas skin interstitial fluid [$NO_3^-$] was lower than in venous plasma. These observations suggest that acute dietary $NO_3^-$ supplementation can increase substrates for oxygen-independent NO synthesis in the skin interstitial fluid which may have implications for regulating physiological responses in human skin and surrounding areas.

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**Figure legends**

**Figure 1.** A schematic of the experimental protocol.

**Figure 2.** Individual and average [$NO_3^-$] and [$NO_2^-$] for both the skin interstitial fluid and venous plasma measured at baseline. Data (mean ± standard deviation) are pooled from both the BR and PL groups (n = 16).

**Figure 3. [**$NO_3^-$] and [$NO_2^-$] in the skin interstitial fluid (circles) and venous plasma (triangles) at baseline and after drinking BR (panels A, D). △[$NO_3^-$] or △[$NO_2^-$] from baseline was compared between the skin interstitial fluid and venous plasma (panels B, E). Area under the curve above baseline over 4-h post-consumption period in the skin interstitial fluid and venous plasma was compared for [$NO_3^-$] or [$NO_2^-$] (panels C, F). Data are presented in mean ± standard deviation (n = 10 for all figures). *, $P < 0.001$ for the skin interstitial fluid vs. plasma.

**Figure 4.** Correlation analysis between the skin interstitial fluid and venous plasma for [$NO_3^-$] or [$NO_2^-$] using all data points after drinking BR (panel A, B, n = 40).

**Figure 5.** Blood pressures (panels A-C) at baseline and after drinking BR. Data are presented as mean ± standard deviation (n = 11 for all figures).

**Figure S1.** Correlation analysis between the skin interstitial fluid and venous plasma for [$NO_3^-$] (panels A-D) or [$NO_2^-$] (panels E-H). Data were analyzed at 1, 2, 3, and 4 h after drinking BR (n = 10 for each figure).

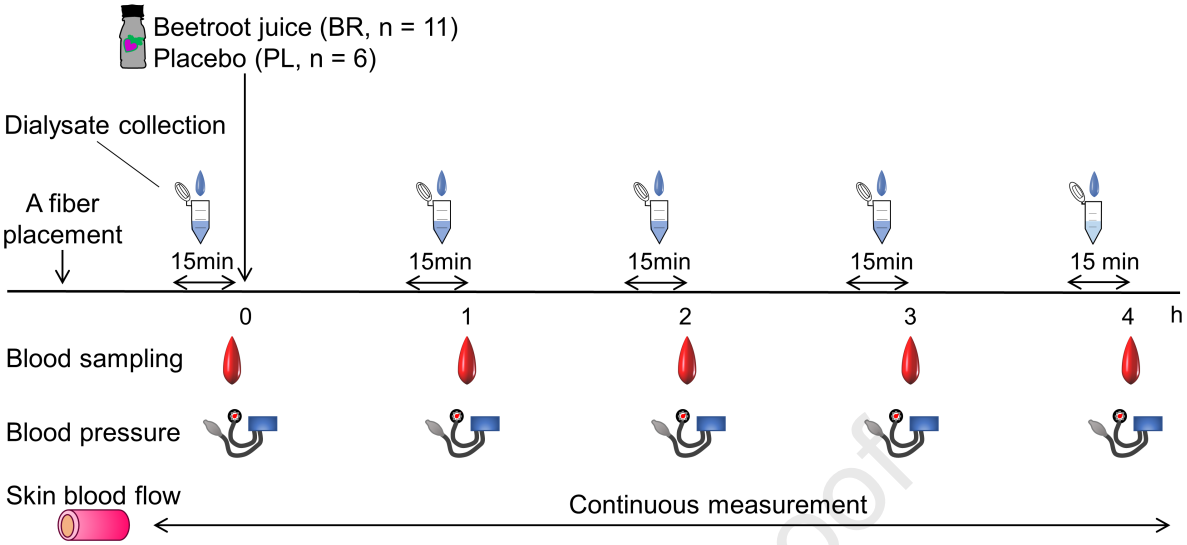
**Figure S2.** Skin blood flow index from a representative participant who ingested BR.

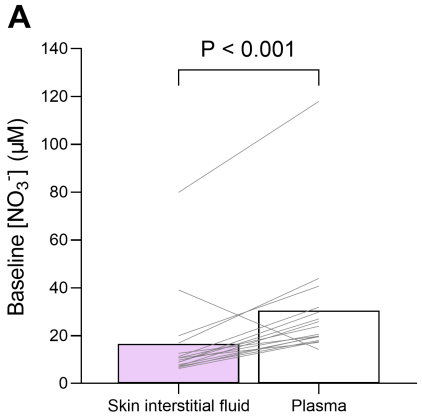
**Figure S3.** Skin blood flow index (panel A) and skin vascular conductance (panel B) at baseline and after drinking BR. Data are presented as mean ± standard deviation (n = 11 for all figures). †, $P \leq 0.045$ for baseline vs. post consumption period.

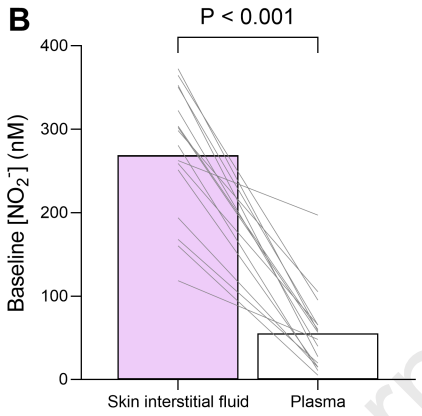
**Figure S4**. [$NO_3^-$] and [$NO_2^-$] in the skin interstitial fluid (circles) and plasma (triangles) at baseline and after drinking PL juice (panels A, B). Data are presented in mean ± standard deviation (n = 6 for all figures). Statistical analyses were not performed due to small sample size except generating mean and standard deviation.

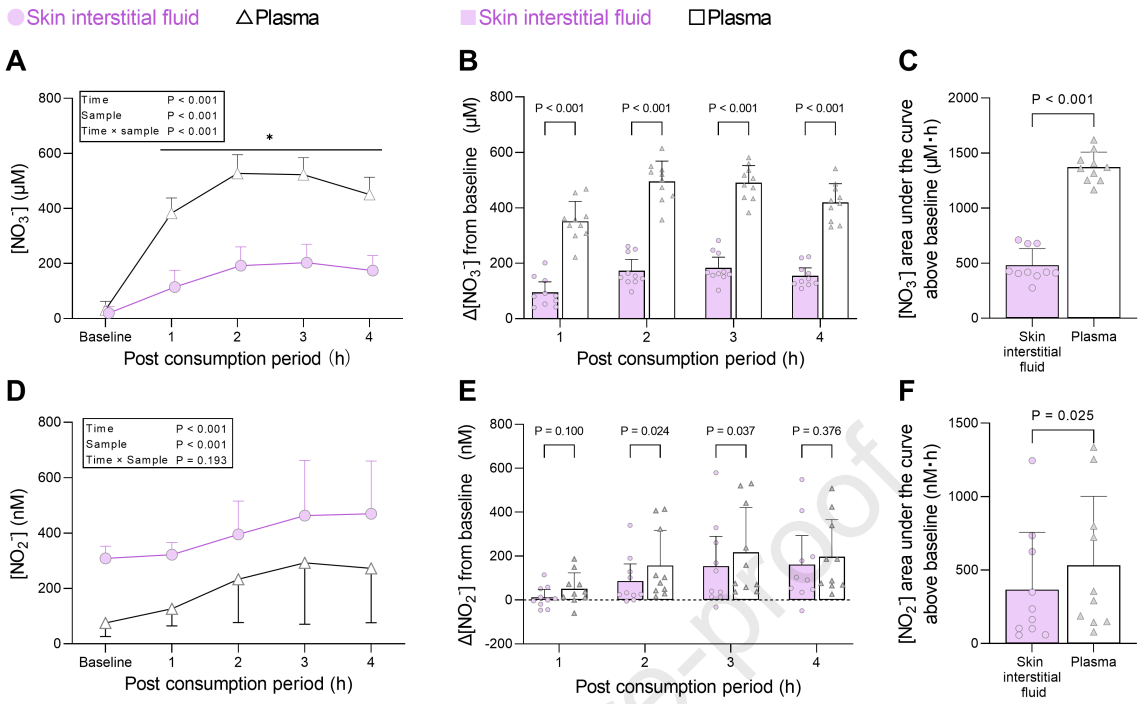
**Figure S5**. Blood pressures (panels A-C), skin blood flow index (panel D), and skin vascular conductance (panel E) at baseline and after drinking PL juice. Data are presented in mean ± standard deviation (n = 6 for all figures). Statistical analyses were not performed due to small sample size except generating mean and standard deviation.

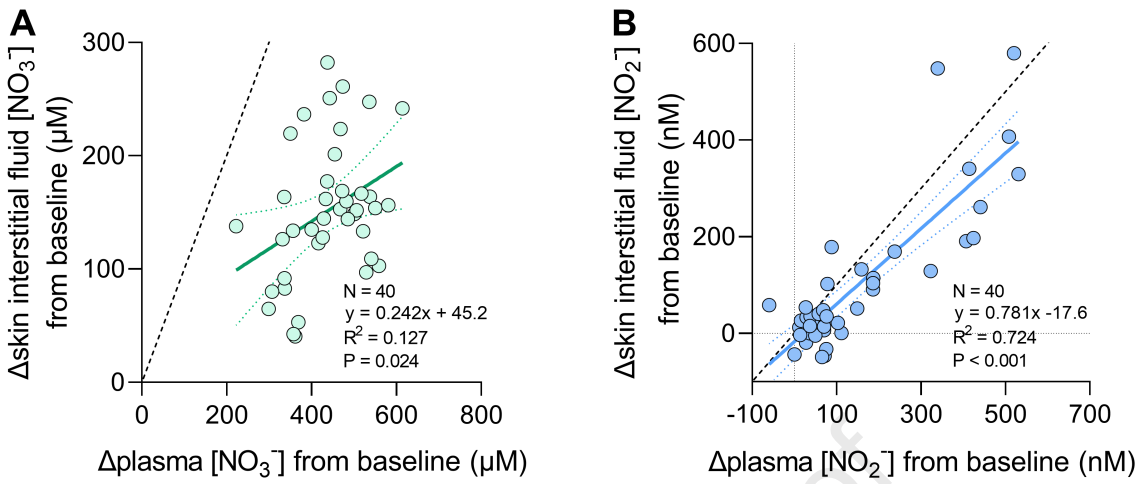
**Figure S6.** A summary figure describing how dietary $NO_3^-$ is absorbed and metabolized, and potentially influences functions in human skin and exercise performance.

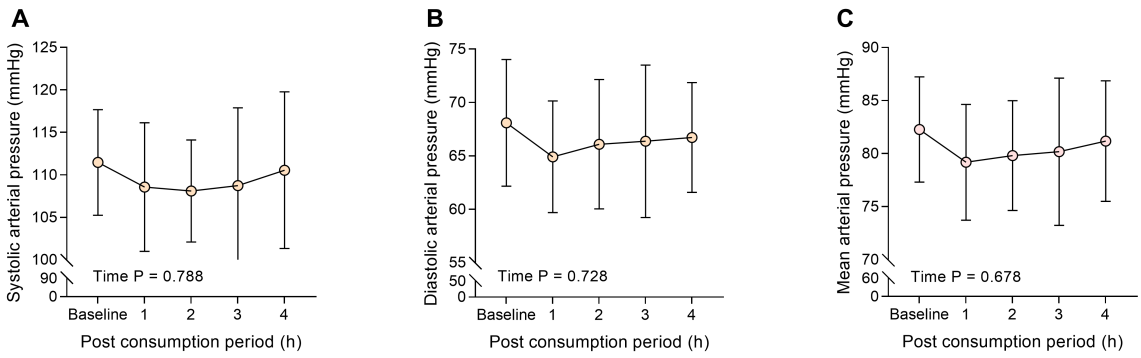










Highlights

- $[NO_3^-]$ and $[NO_2^-]$ in skin interstitial fluid was assessed using microdialysis.
- Basal $[NO_3^-]$ is lower in skin interstitial fluid relative to plasma.
- Basal $[NO_2^-]$ is higher in skin interstitial fluid compared with plasma.
- Dietary nitrate ($NO_3^-$) increases $[NO_3^-]$ and $[NO_2^-]$ in skin interstitial fluid.