Supplemental Protein and a Multi-Nutrient Beverage Speed Wound Healing Following Acute Sleep Restriction in Healthy Adults[1]

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Abbreviations:

ANAM, the Automated Neuropsychological Assessment Metrics
ANOVA, analysis of variance
AUC, area under the curve
BMI, body mass index
CRP, C-reactive protein
DEXA, dual energy x-ray absorptiometry
CD, clusters of differentiation
EEE, exercise energy expenditure
ELISA, enzyme linked immunosorbent assay
GH, growth hormone
GMFI, geometric mean fluorescence intensity
HLADR, human leukocyte antigen DR isotype
HMB, β-hydroxy-β-methylbutyrate
IL, interleukin
MDRIs, Military Dietary Reference Intakes
METS, metabolic equivalents
MEQ, Morningness/Eveningness questionnaire
NUT, participants consumed a controlled, isocaloric diet with 1.50 $g \cdot kg^{-1} \cdot d^{-1}$ protein plus multi-nutrient beverage (NUT; L-arginine: 20.0 $g \cdot d^{-1}$, L-glutamine: 30.0 $g \cdot d^{-1}$, omega-3 fatty acids: 1.00 $g \cdot d^{-1}$, zinc sulfate: 24.0 $mg \cdot d^{-1}$, cholecalciferol: 800 $IU \cdot d^{-1}$ and vitamin C: 400 $mg \cdot d^{-1}$)
PLA, participants consumed a controlled, isocaloric diet with 0.900 $g \cdot kg^{-1} \cdot d^{-1}$ protein plus placebo
PSQI, Pittsburgh Sleep Quality Index
SIgA, secretory immunoglobulin A
TEE, total energy expenditure
TEWL, trans-epidermal water loss
USARIEM, United States Army Research Institute of Environmental Medicine

ABSTRACT

Background. Physiological and psychological stress slow healing from experimental wounds by impairing immune function.

Objective. To determine whether supplemental protein and multi-nutrient supplementation improve wound healing markers following acute stress induced by acute sleep restriction.

Methods. In this single-blind, cross-over study of generally healthy young adults (18 males/2 females; 19.7±2.30 years [mean±SD]), experimental wounds were created by removing the top layer of forearm blisters induced via suction after 48-h of 72-h sleep restriction (2-h nightly sleep), a protocol previously shown to delay wound healing. Skin barrier restoration (measured by trans-epidermal water loss, TEWL) assessed wound healing up to 10 days post-blistering, and local immune responses were evaluated by serial measurement of cytokine concentrations in fluid collected at wound sites for 48-h post-blistering. Participants consumed controlled, isocaloric diets with either 0.900 $g \cdot kg^{-1} \cdot d^{-1}$ protein plus placebo (PLA) or 1.50 $g \cdot kg^{-1} \cdot d^{-1}$ protein plus multi-nutrient beverage (NUT; L-arginine: 20.0 $g \cdot d^{-1}$, L-glutamine: 30.0 $g \cdot d^{-1}$, omega-3 fatty acids: 1.00 $g \cdot d^{-1}$, zinc sulfate: 24.0 $mg \cdot d^{-1}$, cholecalciferol: 800 $IU \cdot d^{-1}$ and vitamin C: 400 $mg \cdot d^{-1}$) during sleep restriction and for 4 days afterwards.

Results. Skin barrier restoration (primary outcome) was shorter for NUT [(median, interquartile range) (3.98, 1.17 days)] compared to PLA (5.25, 1.05 days) (P=0.001). Cytokines from wound fluid (secondary outcome) increased over time (main effect of time $P \leq 0.001$), except IL-13 (P=0.07); however, no effects of treatment were observed.

Conclusions. Supplemental nutrition may promote wound healing following sleep restriction in healthy adults including military personnel, the latter of which also have a high incidence of

wounds and infection.

Keywords: sleep insufficiency; cytokines; immune function; tissue damage; tissue healing; stress; military personnel; first responders

INTRODUCTION

Physiological and psychological stress can degrade immune responsiveness (1), promote inflammation (2-4), and reduce T-cell and natural killer cell function (5-8). These effects are seen following exposure to both single stressors (e.g., acute and chronic sleep restriction) as well as following exposure to combinations of different stressors such as high exercise volume, suboptimal sleep, suboptimal nutritional intake, and stress or anxiety. Chronic and acute stress deriving from single or multiple stressors can consequently increase risk of illness and infection, and impair wound healing (9-16).

Wound models that perturb the skin barrier can be used to assess functional status of the immune system, and provide insight into aspects of immune function that other in-vitro and in-vivo models do not fully capture, such as innate immune and pro-inflammatory responses and tissue remodeling. For example, our group and others have shown that a suction blister wound model is a useful tool for studying immune responsiveness in populations exposed to a variety of stressors and for testing the efficacy of countermeasures to promote or enhance recovery (17). The model involves creating superficial wounds, which are used to directly assess the functional status of the innate immune system (i.e., the ability to heal from a wound), and to gain insight into local pro-inflammatory responses and the tissue remodeling process. Our group has shown that the suction blister method is sufficiently reliable for assessing skin barrier restoration, a

proxy measure of wound healing rate, and local immune responsiveness at the site of experimental skin wounds (18).

Consuming a diet that meets energy demands while providing adequate macronutrients and micronutrients promotes all aspects of immune function (11-16, 19-21). In contrast, inadequate intakes of energy, vitamins A, D or C, folic acid, zinc, iron, or selenium negatively affect immune function and decrease resistance to infections (15, 22-26). Additionally, in the context of wound healing, proteins are a vital component of collagen synthesis, and consuming additional protein beyond the current dietary recommended intake (DRI; 0.8 g protein · kg^{-1} body weight · day^{-1}) may mitigate stress-induced decrements in immune function and wound healing (19, 20, 27, 28). In adults undergoing major elective surgery, clinical nutrition support guidelines recommend enteral formulations with arginine, glutamine, omega-3 fatty acids, and antioxidants to optimize immune function and recovery time (19, 20, 27). Vitamin C is also important for wound healing, playing roles in collagen synthesis, fibroblast proliferation, capillary formation and neutrophil activity (20), while omega-3 fatty acids enhance T-cell and natural killer cell activity and reduce systemic inflammation (29, 30). Additionally in otherwise healthy adults, arginine contributes to collagen deposition and cellular growth, and impacts microcirculation by increasing the production of nitric oxide (19, 28, 31), while glutamine stimulates the proliferation of fibroblasts, subsequently contributing to wound closure (19). However, the efficacy of these nutrients in combination for modulating immune function and promoting healing in healthy individuals who are immune-compromised as a result of exposure to physical or cognitive stressors (e.g., sleep restriction) has received little attention.

Addressing that gap is relevant for populations such as military personnel who frequently experience immune-compromising physical and cognitive stressors with inadequate energy and

nutrient intake. Our group recently reported that a military-relevant stressor (i.e., relatively severe, sleep restriction to 2 h sleep per night over 72-h compared to 7-9 h sleep per night) delayed wound healing assessed using the suction blister model by approximately one day, and that supplementation of a higher protein diet with arginine, glutamine, vitamin C, vitamin D, zinc and omega-3 fatty acids mitigated some decrements in local immune responses as evidenced by altered cytokine responses at wound sites (17). Although no statistically significant effect of nutrition supplementation on wound healing rate was observed in that study, our inability to detect such an effect may have reflected a type 2 error given that participants exhibited higher than expected inter-individual variability in healing rate.

The current study was designed to overcome that potential limitation. To reduce inter-individual variability and increase power, we employed a placebo-controlled, crossover study design and implemented stricter dietary control during the current study. Otherwise, the nutrition intervention and experimental conditions were the same, so as to determine whether multi-nutrient supplementation would improve skin barrier restoration in response to acute sleep restriction (i.e., 72-h sleep restriction with 2-h nightly sleep). We hypothesized that, relative to placebo, when supplemental nutrition was consumed during and after sleep restriction, cytokine response at the wound site would be preserved (i.e., as indicated by a higher pro-inflammatory response and a lower anti-inflammatory response during the early phase of wound healing) and skin barrier restoration would be shorter.

MATERIALS AND METHODS

Study Design

This study used a single-blind, placebo-controlled, cross-over design, wherein all participants underwent two separate conditions, first, the placebo (PLA) treatment (0.900 g protein · kg^{-1} body weight · day^{-1} with placebo beverage) with sleep restriction and; second, the experimental (NUT) treatment (1.50 g protein·kg^{-1} body weight·day^{-1} with a multi-nutrient beverage) with sleep restriction. In this design, sleep opportunities were restricted to 2 h (~0430-0630) every 24 h period for 3 consecutive days (*Figure 1*). This design was chosen based on our previous work which demonstrated that this sleep restriction model delayed initial restoration of the skin barrier by ~1 day (17), as measured by trans-epidermal water loss (TEWL) measurements described below. Dietary protein levels were selected to represent the lower and higher ends of the Military Dietary Reference Intakes (MDRIs) [Army Regulation 40-25, OPNAVINST 10110.1/MCO10110.49, AFI 44-141]. For all participants, the NUT trial always followed the PLA trial with a 2 week washout period between study trials. The order of PLA and NUT was not randomized because our study participants (active-duty military personnel) were not available for the amount of time needed to achieve adequate nutrient washout following NUT. Blisters were applied after 48 h of sleep restriction; alternate arms were blistered during PLA and NUT, and the order was randomly assigned. Primary outcomes included skin barrier restoration (measured by skin vapor permeability) and wound inflammatory responses. Prior to beginning data collection the study was registered on Clinicaltrials.gov as trial number NCT03525184.

Participants

Participants were military personnel assigned to Natick Soldier Systems Center, Natick, MA. Fifty-four percent (n = 20) of the 37 study participants completed data collection and were included in the data analyses. Three participants withdrew due to relocation from the geographical area; six participants were withdrawn due to unrelated medical issues; seven participants changed their mind about participating (e.g., due to the time commitment, weekend data collection and/or exercise restrictions); and one participant was withdrawn during the sleep restriction period for not complying with study instructions (i.e., unable to stay awake).

Data collection occurred from February 2018 to December 2019 at the U.S. Army Research Institute of Environmental Medicine (Natick, MA). Each volunteer gave their written, informed consent after a verbal explanation of the study. Individuals were included if they were between the ages of 17 and 45 years, were generally healthy and not taking medications, were not pregnant or lactating, had no history of psychiatric disorder requiring hospitalization or psychiatric medication usage within the past three years, and routinely slept between 7 and 9 hours per night at least five days per week. Females were eligible to participate if they were not taking hormonal contraceptives or if they were on continuous hormonal contraceptives which maintain continuous estrogen/progesterone levels throughout the 28-day cycle.

All subjects completed an initial screening and were medically cleared for participation. The study was approved by the Institutional Review Board, United States Army Research Institute of Environmental Medicine, Natick, MA. The investigators adhered to the policies for protection of human subjects as prescribed DOD Instruction 3216.02 and the research was conducted in adherence with the provisions of 32 CFR Part 219.

Research procedures

Assessment of Habitual Sleep Patterns

All participants self-reported that they regularly slept 7-9 hours per night prior to beginning the study. General sleep patterns were assessed during the baseline testing period (T-5 days, *Figure 1*) via the Morningness/Eveningness questionnaire (MEQ), Pittsburgh Sleep Quality Index (PSQI), actigraphy, and a paper-and-pencil sleep diary. The MEQ (32) is a 19-item questionnaire that assesses respondent's circadian preference, sleep-wake pattern for activity, and morning and evening alertness. The PSQI is a 19-item questionnaire and assesses self-reported sleep quality in adults (33). To remain in the study, participants needed to score between 31 and 69 on the MEQ or ≤5 on the PSQI, thus avoiding extremes in "morningness" or "eveningness" and poor sleep quality, with the latter being indicated by higher PSQI scores.

Participants donned a wrist-worn actigraphy monitor (Actiwatch Spectrum Plus, Philips Respironics, Murrysville, Pennsylvania or an equivalent) for seven consecutive days immediately prior to each sleep restriction period to verify that they had slept between 7 and 9 hours per night. Participants also maintained a paper-based sleep diary, in which they recorded the time they went to bed (with the intent to sleep) and the time they awoke each day.

Assessment of Life Stressors

The Perceived Stress Scale (34) was administered to all participants within a week of the first sleep restriction period. This scale is a widely use, reliable and valid 14-item self-report measure of perceived stress, wherein respondents rate the stressfulness of their life during the previous month with higher sum scores indicating greater perceived stress.

Anthropometrics

Standing height and weight was measured at the beginning of the study and daily during

the live-in portion of the study (weight only) as described elsewhere (17) using a stadiometer (Seritex, Inc., Carlstadt, NJ) and a calibrated electronic scale (Tanita WB-110A Class III, Tokyo, Japan). Body composition (i.e., total body mass, fat-free mass, and fat mass) was also measured at baseline, using dual energy x-ray absorptiometry (DEXA, DPX-IQ, GE Lunar Corporation, Madison, WI), wherein fasted participants laid face-up on the DEXA densitometer table in shorts, t-shirts, and stocking feet.

Menstrual Cycle Interview

Female participants were interviewed regarding oral contraceptive use and the timing of their menstrual cycle to determine length of menstrual cycle, in order to schedule live-in testing during the luteal phase (i.e., day ~14-28) for participants not on oral contraceptives.

Sleep Restriction

Study participants arrived to the laboratory the day before the sleep restriction period began and slept (~8 h) overnight at the laboratory. Sleep restriction was conducted across 3 consecutive days, during which participants slept 2 hours each 24 h cycle (~0430-0630). Participants were continuously monitored throughout the sleep restriction period and, when not sleeping, engaged in a variety of supervised activities to maintain wakefulness (e.g., exercise, video games, television, movies). Additionally, participants wore an actigraphy monitor (described earlier) during, and for five days after, each sleep restriction period. This sleep restriction model was selected based on the somewhat typical wake-sleep pattern that military personnel experience during training (35) and combat missions (36). The sleep-wake pattern in this study is also relevant to non-military emergency service personnel and medical interns, who also encounter occupational scenarios where sleep restriction is unavoidable (37-39); and,

endurance athletes who may self-impose sleep restriction during short-term, multi-day events (40-42).

Assessment of Tiredness and Fatigue

Reported subjective tiredness and fatigue was assessed at the beginning (Day 1, ~1100) and towards the end (Day 3, ~0200) of the sleep restriction period using The Automated Neuropsychological Assessment Metrics (ANAM) Sleepiness Scale and Mood Scale, respectively, wherein higher values indicate higher sleepiness and fatigue (43).

Determination of Total Daily Energy Expenditure

Total Energy Expenditure (TEE) (44) during the sleep restriction period was estimated using metabolic equivalents (METS) to determine the energy intake required to maintain body weight for each participant during the sleep restriction period, as described previously (17, 44, 45).

Physical Activity

Mild to moderate physical activity (e.g., treadmill walking, outdoor walking and cycle ergometry) was prescribed during the sleep restriction period to maintain wakefulness and sustain the participants' habitual level of energy expenditure. As such, exercise energy expenditure (EEE; kcals·d^{-1}), derived from exercise recall interviews and added to the TEE equation, was used to determine the amount of mild to moderate physical activity prescribed. Estimates of exercise workloads and energy expenditure were calculated as described elsewhere (44) and workload was monitored as described previously (46).

Assessment of Dietary Intake

Intake of omega-3 fatty acid-rich foods, probiotics, other dietary supplements (including multi-vitamin/minerals) and oral antibiotic use was assessed at baseline by questionnaire, and

participants were asked to refrain from consuming these items for the duration of the study. Approximately two weeks before the first experimental period (PLA), volunteers completed 3-day food records to determine their typical protein consumption in order to better instruct them on how to achieve the prescribed protein intake (see "*Study Diet*"). Additionally, participants recorded all foods and beverages consumed for 3 days prior to each sleep restriction period, and for 5 days following each sleep restriction period to allow quantification of energy, macronutrients, and nutrients affecting immune function. Food records were reviewed daily and finalized for accuracy by Registered Dietitians, and analyzed for nutrient content using computer-based nutrient analysis software (Food Processor, ESHA Research, Salem, OR).

Study Diet

Before sleep restriction period: In the five consecutive days leading up to the first sleep restriction period (PLA), participants consumed measured and provided foods that contained ~0.900 g protein·kg^{-1} body weight·day^{-1}. In the five consecutive days leading up to the second sleep restriction period (NUT), participants were instructed to consume ~1.50 g protein · kg^{-1} body weight · day^{-1}, but foods were not provided. Prior experience with this study population [17] indicated that habitual protein intake tends to be consistent with the higher protein prescription, thus providing some measure of confidence that participants would comply. Conversely, based on prior experience, compliance was not expected with the lower protein prescription, thus a lead-in diet was provided prior to PLA but not NUT. These protein intake prescriptions mimicked the protein prescription during the sleep restriction periods in order to habituate liver enzymes. Participants met with a trained study Registered Dietitian every 1-2 days to assess compliance with protein intake.

During sleep restriction period (Figure 1): During both sleep restriction periods, participants were provided measured diets to consume that were designed to maintain energy balance. Study diets included commercially-available food items and water was allowed *ad libitum*. Diets were designed by Registered Dietitians to provide either ~0.900 g protein·kg body weight^{-1}·day^{-1} during PLA or ~1.50 g protein·kg body weight^{-1}·day^{-1} during NUT. Diets were approximately matched between PLA and NUT for fat, arginine, glutamine, vitamin C, vitamin D, zinc and omega-3 fatty acids (excluding contributions from the multi-nutrient beverage), while protein and carbohydrates differed between treatments. This protein range represents the lower and higher ends of the MDRI (0.800 – 1.600 g·kg body weight^{-1}·day^{-1}) and are approximately at or double the RDA for protein (0.800 g·kg body weight^{-1}·day^{-1}), respectively. The higher level of protein was chosen for the intervention diet based on general recommendations for immune-supporting diets (19, 20, 27, 28). Differences in protein content of the diet between PLA and NUT, and the study hypothesis that additional protein may improve immune recovery, were not emphasized in the consent form or the oral participant briefing. Food items were generally the same between PLA and NUT (cereal, entrees, pretzels, apple sauce, sports drinks, etc.), with additional sources of protein (e.g., string cheese and beef jerky) during NUT (*Supplemental Table 1*). Some of the food items provided by the study diet were chemically analyzed (Covance Inc., or equivalent) to confirm their composition of macronutrients and select micronutrients (i.e., vitamin C, vitamin D, n-3 fatty acids and/or zinc). Registered Dietitians prepared each participant's daily meals and snacks, and food consumption was monitored by trained study staff. Dietary intake was analyzed for nutrient content using computer-based nutrient analysis software (Food Processor, ESHA Research, Salem, OR). Participants were instructed to refrain from caffeine three days prior to the sleep restriction

period to avoid the effects of caffeine withdrawal during the sleep restriction period, and were not allowed to consume any food or beverages (to include caffeine) other than those provided.

Post-sleep restriction period (Days 4-8, Figure 1): Upon leaving the lab on Day 4, participants were provided with the rest of that day's food/beverages and were instructed to consume a protein-controlled (PLA: ~0.900 g protein · kg^{-1} body weight · day^{-1}; NUT: 1.50 g protein · kg^{-1} body weight · day^{-1}), *ad libitum* diet on days 5-8. Participants were given detailed instructions regarding protein-containing food, beverages, and portion sizes to meet the study's protein guidelines, and food records were reviewed daily by trained Registered Dietitians to confirm compliance.

Multi-Nutrient Beverage

The multi-nutrient beverage contained L-arginine (20.0 $g \cdot d^{-1}$), L-glutamine (30.0 $g \cdot d^{-1}$), omega-3 fatty acids (1.00 $g \cdot d^{-1}$), zinc sulfate (24.0 $mg \cdot d^{-1}$), cholecalciferol (800 $IU \cdot d^{-1}$) and vitamin C (400 $mg \cdot d^{-1}$). The multi-nutrient beverage and placebo are consistent with those provided in our previous study (17) and was originally based on formulas used in clinical settings which have shown benefits related to post-surgical infectious complications (47-51) and wound healing disorders (52). The beverages were consumed twice per day during the sleep restriction period (Days 1-3) and the post-sleep restriction period (Days 4-8). Study team members witnessed beverage consumption during the sleep restriction period and on each morning of the post-sleep restriction period; and, participants consumed the beverage, on their own, each afternoon of the post-sleep restriction period and returned the empty container the following morning.

Micronutrient Status

To determine background nutrient status, vitamin C and 25-hydroxyvitamin D were measured from fasted blood samples collected at the baseline and on the morning of PLA and NUT day 1 using colorimetric (kit #ab65656, Abcam, Waltham, MA) and enzyme linked immunosorbent assay (ELISA) kits (kit #AC-57SF1, Immunodiagnostic Systems, Inc., East Boldon, United Kingdom), respectively. These micronutrients were also measured at the conclusion of NUT (Day 8) to determine differences in circulating concentrations before and after the nutrition intervention.

Assessment of mucosal immunity

Secretory Immunoglobulin A (SIgA) was measured in tear fluid obtained each morning of the live-in study periods to evaluate mucosal immunity. Methods were adapted from Hanstock et al. (53, 54) and Fullard and Snyder (53, 54). Briefly, timed, unstimulated tear samples (~1 µL) were collected from the distal corner of the eye using 10 µL fire-polished glass microcapillary pipettes (Sigma-Aldrich, St. Louis, MO) and sample volume was calculated. SIgA concentration was determined by ELISA (Kit #1602, Salimetrics, State College, PA); and, the product of tear flow rate (i.e., volume collected divided by the collection time, assuming tear density of 1.00 $g \cdot mL^{-1}$) and SIgA concentration determined tear SIgA secretion rate.

Systemic Markers of Inflammation and Immune function via peripheral blood

Whole blood was drawn from an antecubital vein once during the baseline period and daily, upon waking, during the live-in portions of the study. Cortisol, growth hormone (GH), C-reactive protein (CRP), and cytokines were assessed from serum. Cytokines (Kit #HCYTOMAG-60K, EMD Millipore, Burlington, MA) and CRP (Kit # L2KCRP2, Siemens

Healthcare, Erlangen, Germany) were measured, in duplicate, using Multiplex bead based on Luminex® technology (MAGPIX® System, Luminex, Austin, TX, USA). Cortisol (Kit #L2KCO2, Siemens Healthcare, Erlangen, Germany) and GH (Kit #LKGRH1, Siemens Healthcare, Erlangen, Germany) were measured using the Immulite immunoassay system (Siemens Healthcare, Erlangen, Germany).

Suction Blister Induction and Fluid Sampling

Suction blisters were induced after 48-h of the sleep restriction protocol according to previously described methods (18). Briefly, a vacuum pressure was applied to a polycarbonate template on the forearm to separate the epidermis from the dermis, thereby forming a series of eight blisters (*Figure 2*). Blister fluid was subsequently sampled and the top of each blister was removed. Polycarbonate wells (*Figure 2*) were secured over the blisters and the autologous fluid mixture (17), which acts as a soluble chemotactic substance (55), was syringed into the wells. The concentration of inflammatory [interleukin (IL)-1β, IL-6, IL-8, TNF-α, MIP-1α and MIP-1β] and anti-inflammatory (IL1-RA, IL-4, IL-10 and IL-13) cytokines was assessed by removing fluid from distinct wells following blister formation at the following time points (corresponding study day denoted in parentheses, *Figure 1*): 4 h, 7 h (day 3), 24 h (day 4), 48 h (day 5), 72 h (day 6) and 96 h (day 7) following blister formation.

Flow cytometry (flow cytometer: Becton Dickinson, Accuri C6; and FCS Express v7.0, De Novo Software, Pasadena, CA, USA) was used to characterize infiltrating leukocyte populations isolated from autologous exudate from the de-roofed blister sites. Leukocytes were first counted on a glass hemocytometer (Fisher Scientific, Waltham, MA) and assessed for viability via trypan blue exclusion. Subsequently, cells were labeled with anti-human primary fluorophore-conjugated antibodies raised against specific clusters of differentiation (CD) to

facilitate cellular identification. Monoclonal antibodies [(human leukocyte antigen DR isotype) HLADR, CD14, CD16 and CD45] were purchased from ThermoFisher Scientific (Waltham, MA) and conjugates, clones and catalogue numbers for the antibodies are provided in *Supplemental Table 2*. Characteristic light scattering properties (e.g., side scatter; SSC), as well as fluorescently labeled cell surface markers were used to quantify various leukocyte populations**.** Fluorescence staining intensity is presented as geometric mean fluorescence intensity (GMFI).

Transepidermal Water Loss (TEWL) to Assess Skin Barrier Restoration

The time to skin barrier restoration, a proxy measure for wound healing rate, was assessed by measuring TEWL from individual blisters using the VapoMeter (Delfin Technologies Inc., Miami, FL) as previously described (17). Briefly, the skin limits the movement of water in and out of the body. The premise of measuring TEWL to assess skin barrier restoration is based on the fact that removal of the epidermis at the blister site increases water permeability, resulting in higher TEWL values which then decrease over time as the wound heals [18]. TEWL was measured each morning (beginning on day 4, ~24 h after blister formation) from the lower four wound sites and an adjacent, non-wounded, control site. Ultimately, the difference between the TEWL measurements from wound number six and the TEWL at the control site were used to assess skin barrier restoration, since the majority of participants developed a blister at this location (i.e., all participants in PLA, and 18 of 20 participants in NUT) and blister size was consistent between participants at this site during both the current study and our prior work (17). Calculations for determining time to skin barrier restoration were adapted from Keicolt-Glaser et al. (56). The calculation first established a 90% 'Standard of Restoration' using the TEWL values measured on day 4 (~24 h after blister

induction):

[TEWL measurement from wound site – TEWL measurement from control site] x 0.10

To best identify the time point at which the skin barrier was considered "restored," we plotted the difference between TEWL measured at the wound and control sites (y-axis) versus the time since blistering (x-axis) at each measurement time point. We then calculated an exponential line of best fit, $y = ae^{bx}$, using the exponential regression function in Microsoft Excel (Excel 2016, Microsoft, Redmond, WA)]. The equation was then solved for x after setting y equivalent to the participant's 90% 'Standard of Restoration'.

Calculations and Statistical Analyses

The primary dependent variable of interest was skin barrier restoration rate, and secondary variables of interest were cytokine concentrations from the wound fluid. Autologous fluid was not well retained in the polycarbonate wells (i.e., due to poor adherence of the adhesive on the skin and subsequent leakage) on days 6 and 7 (i.e., 72-h and 96-h), thus outcomes related to those time-points were excluded from subsequent analyses. Sample size calculations (two-tailed paired t-test) were based on data from our previous parallel-arm study in which NUT improved time to skin barrier restoration relative to placebo by a mean of 0.5 days (SD = 0.8). Using a lower SD of 0.7 days due to the expected reduction in variability resulting from the crossover design, we determined that 19 participants were required to detect a 0.5 d difference in time to skin barrier restoration using a paired t-test and alpha = 0.05 and power 0.80. With regard to wound cytokine concentrations, sample size was estimated (two-tailed paired t-test) based on area under the curve calculated from IL-8 and IL-6 concentration measured at 4-, 7-, and 24-h post-blistering during our prior study [17]. The mean ± SD difference between treatments was 5.01 ± 2.00 $pg \cdot mL^{-1}$ for IL-8 and 2.51 ± 2.00 $pg \cdot mL^{-1}$ for IL-6. Using those mean difference and SDs, 19 participants were determined to be sufficient to detect a 1.40 $pg \cdot mL^{-1}$ difference or 28%

difference in IL-8 and 56% difference in IL-6 responses between treatments at $\alpha = 0.05$ and power = 0.80.

All statistical analyses were conducted using the IBM SPSS statistical package version 19.0 (IBM Inc., Armonk, New York). Data were examined for outliers both quantitatively and graphically, and normal distribution of data was confirmed via the Shapiro-Wilk test. Data that were not normally distributed were log transformed (log10) (i.e., cytokine wound and serum concentrations, and GH, cortisol, CRP and SIgA) or non-parametric testing was used if normal distribution was not achieved by log transformation. Repeated measures analysis of variance (ANOVA) was used to assess changes in body weight and dietary intake, over time (i.e., daily). Due to non-normal distribution, Wilcoxon Signed Rank Tests were used to assess between-treatment differences for time to skin barrier restoration and sleepiness and fatigue scores. Paired samples t-tests were used to compare vitamin C and D status before and after consuming the nutrition intervention (i.e., day 1 and day 8, respectively of NUT; *Figure 1*). Pearson correlation coefficient was used to assess the relationship between the change in serum vitamin status and the difference in skin barrier restoration between treatments (i.e., PLA and NUT). Linear mixed models with first order autoregressive covariance type were used to determine main effects of time and treatment (NUT and PLA), and their interaction on the following variables: wound fluid cytokine concentration (i.e., 4, 7, 24 and 48 h post-blistering, *Figure 1*), serum cytokine concentrations (i.e., day 1, 2, 3 and 4, *Figure 1*), concentrations of CRP, GH, cortisol, SIgA (i.e., day 1, 2, 3 and 4, *Figure 1*), and cell counts and GMFI (i.e., 24 and 48 h post-blistering). Linear mixed models were also used to assess between-treatment differences in time to initial skin barrier restoration, controlling for post intervention daytime sleep. Adherence to model assumptions was confirmed for all models. When the interaction term was not statistically

significant, the term was removed from the model in order to interpret main effects. Post-hoc testing (i.e., t-tests of marginal means), with Bonferroni correction to control the familywise error rate, was conducted to examine within- and between-treatment differences only when interactions were significant. After dropping any non-significant time by treatment interactions, post-hoc testing between timepoints was conducted to determine the effects of time. Results are presented as estimated marginal means (± SEM) unless otherwise noted. A two-tailed p-value of less than 0.05 was considered statistically significant.

RESULTS

Baseline Measurements

Twenty participants completed the study and were included in the final data set; and, subject characteristics at study entry are provided in *Table 1*. The two female participants were not taking continuous oral contraceptives, thus they underwent live-in testing during the luteal phase of their successive menstrual cycles. The dietary intake survey confirmed that participants did not habitually consume omega-3 fatty acid-rich foods, probiotics or other dietary supplements prohibited by the study protocol. Mean average nightly sleep duration leading up to the live-in testing was not different between PLA and NUT (8.11 ± 0.61 and 7.91 ± 1.00 h, P = 0.5).

Anthropometrics, Energy Expenditure & Dietary Intake

Average TEE for the total study group (i.e., NUT and PLA) was 2717 ± 483 kcals·d^{-1}, with EEE contributing 406 ± 202 kcals·d^{-1}. There were no within or between treatment differences in body weight change [(mean difference D1-D4 ± SD) PLA: 0.130 ± 1.31 kg and NUT: 0.123 ± 1.31 kg, P = 1.0], indicating that participants were in energy balance.

Table 2 shows the dietary intake characteristics of participants during PLA and NUT.

There were no differences in energy intake before, during or after the 72-h live-in periods. As planned, dietary intake of protein was higher before and during the NUT treatment compared to before and during PLA, respectively. While protein intake (total grams and g · kg-^{1} body weight · day^{-1}) in the 4 days following the live-in periods was not significantly different between treatments (Table 2), average protein intake from the live-in and post live-in period combined was higher during NUT compared to PLA [(mean ± SD) 1.413 ± 0.288 g protein·kg body weight^{-1}·day^{-1} and 1.074 ± 0.283 g protein·kg body weight^{-1}·day^{-1}, respectively; paired t-test, *P*<0.0001]. No significant time by treatment interactions were noted for either carbohydrate or fat intake. However, after dropping the time by treatment interaction from the model, significant main effects of treatment were noted for carbohydrate intake, with values being lower for NUT compared to PLA.

Reported compliance with the micronutrient beverage prescription during and after the sleep restriction period was 98.8% and 100%, respectively. Two participants did not consume the placebo beverage on the afternoon of Day 4 during PLA. Intakes of arginine, vitamin C, vitamin D and zinc were all higher during and after the NUT live-in study period (P < 0.02), reflecting the nutrient supplementation provided by the study beverage. There was a tendency for the same finding for omega-3 fatty acid intake (P = 0.060).

Micronutrient status

The mean baseline serum concentration of 25-hydroxyvitamin D and vitamin C are shown in Table 1. There was no difference in serum concentrations of 25-hydroxyvitamin D before and after NUT (21.3 ± 6.20 and 22.1 ± 6.01 ng·mL^{-1}, P = 0.27). However, there was a significant increase in serum concentrations of vitamin C during NUT [20.5 ± 8.23 versus 27.4 ± 9.89 µmol·L^{-1} (or 0.361 ± 0.143 versus 0.483 ± 0.174 mg·dL^{-1}), respectively, P = 0.02]. There

was a tendency for a correlation between higher vitamin C concentrations and shorter healing time (R = -0.4, P = 0.10).

Sleep During and After Sleep Restriction

There was no difference between PLA and NUT for average duration of nightly sleep during (2.13 ± 0.183 h and 2.05 ± 0.133 h, respectively) and in the five days following (7.71 ± 1.39 h and 7.59 ± 1.21 h, respectively) the live-in period. However, participants' average duration of daily sleep in the five days following the live-in period was longer for NUT compared to PLA (2.02 ± 0.733 h and 1.45 ± 1.07 h, respectively; mean difference 0.583 ± 0.950 h, P = 0.03).

Tiredness and Fatigue

Subjective perceptions of tiredness and fatigue were not different before or at the end of each sleep restriction period (*Supplemental Table 3*).

Skin Barrier Restoration

There were no significant differences between PLA and NUT with regard to the amount of time taken to raise the blisters (143 ± 37.0 and 149 ± 40.0 min, respectively) or blister area (30.9 ± 11.7 mm and 27.3 ± 11.5 mm, respectively). Time to skin barrier restoration (*Figure 3*) was shorter for NUT (median: 3.98 days, range: 4.32 days) compared to PLA (median: 5.25 days, range: 5.20 days) (P = 0.001), and that treatment effect was still significant (P = 0.001) when average duration of daily sleep in the five days following the live-in period was included as a covariate in the model.

Assessment of mucosal immunity

Baseline SIgA flow rate, concentration and secretion rate (*Supplemental Figure 1*) were not significantly different between PLA and NUT. No treatment or time by treatment

differences were noted in the analysis of SIgA concentration. After dropping the interaction, there was a significant main effect of time indicating that SIgA increased regardless of treatment.

Systemic Markers of Inflammation and Immune Function via Peripheral Blood

There were no within or between treatment changes for any of the measured serum cytokine concentrations (*Figure 4A-F*). Serum concentrations of cortisol (*Figure 4G*) and GH (*Figure 4H*) decreased and increased over time, respectively, irrespective of treatment, but there were no treatment by time interactions. No within or between treatment differences were observed for CRP (*Figure 4I*).

Local immune response (via autologous wound fluid)

Autologous wound fluid was available from 16 of the 20 participants. Of all possible wound fluid collection chambers (n = 309, PLA and NUT combined, to include only those chambers corresponding to viable wound sites), cytokine values from 11% were excluded from serial time-point analyses since less than 65% of autologous serum added to the chambers immediately post-blistering was recovered from these wells at the follow-on time-points. All measured cytokine concentrations (*Figure 5A-I*), except IL-13 (*Figure 5J*) exhibited a main effect of time independent of treatment, increasing, with no treatment or time x treatment differences.

Leukocyte populations (*Supplemental Figure 2*) were not detectable at 7-h post blistering. Independent of treatment, there was a significant decrease over time from day 4 (24-h post-blistering) to day 5 (48-h post-blistering) in both the total proportion of CD45+ lymphocytes (*Supplemental Figure 2A*) as well as proportion of CD45+ contributed by CD16+ monocytes (*Supplemental Figure 2B*). Additionally, there was a significant increase over time, independent of treatment, from day 4 to 5 in the proportion of CD45+ lymphocytes contributed CD14+/HLADR+ (*Supplemental Figure 2C*) and both “classical” (*Supplemental Figure 2D*,

CD14+/CD16-/HLADR+) and "non-classical" (CD14+/CD16+/HLADR+, *Supplemental Figure 2E*) monocytes. Independent of time, significant treatment effects were noted for various cell populations (*Supplemental Figure 2F-K),* wherein expression of HLADR (as determined by GMFI) were higher for PLA compared to NUT. In contrast, the total proportion of CD45+ lymphocytes contributed by CD14-/CD16+ was higher for NUT compared to PLA, regardless of time (*Supplemental Figure 2L*). Time by treatment interactions were observed for the proportion of CD45+ lymphocytes contributed by CD14+ and CD14+/CD16- monocytes, wherein proportions were higher at 24-h post-blistering during PLA compared to NUT (*Supplemental Figure 2M* and *Supplemental Figure 2N,* respectively).

DISCUSSION

Stressors, such as acute sleep restriction, delay healing time and depress the local inflammatory response at wound sites during the initial phases of wound healing. Results of this study confirmed our hypothesis that time to skin barrier restoration following wound induction is shorter when participants consume higher protein and a twice daily multi-nutrient beverage during and after 72-h of sleep restriction compared to lower dietary protein and a placebo beverage. However, in contrast to our previously reported observations (17), the local immune response at the wound sites was not mitigated by the nutrient intervention. Nonetheless, the findings from the current study combined with our earlier findings (17) strongly suggest that higher protein and multi-nutrient supplementation is beneficial to improving time to initial skin barrier restoration in healthy adults consequent to an immune-degrading stressor.

Our previous work demonstrated that 72-h of sleep restriction delayed skin barrier restoration by one day compared with normal sleep (17), and the current study extends those findings by demonstrating that the nutrition intervention mitigated that delay. Of note, skin barrier restoration in response to the sleep restriction in our prior and current studies are similar

(5.00 ± 0.90 and 5.48 ± 1.30 days, respectively), and both are longer than skin barrier restoration following a period of normal sleep (4.20 ± 0.90) (17). Previously, we did not detect significant differences in skin barrier restoration time between PLA and NUT despite the same sleep restriction paradigm and supplementation strategy as the current study. However, the former study did not use a crossover design and higher than expected inter-subject variability in outcome measures and potential confounding variables that were not measured (e.g., sleep duration following sleep restriction, inter-subject differences in responses to sleep restriction) may have prevented detecting the nutrient effect on skin barrier restoration.

In the current study, cytokine responses at the wound site were measured to gain insight into possible mechanisms underpinning the expected nutrition-mediated improvements in skin barrier restoration. Cytokines predictably increased over time regardless of treatment reflecting the inflammatory response to the early phase of wound healing; however, treatment-by-time effects were not detected. Those results contrast with our previous study wherein we observed differences in area under the curve (AUC) of cytokine responses at the wound site (17), which were suggestive of an enhanced early wound healing response with nutrition supplementation. Unfortunately, methodological limitations prevented us from calculating AUC in the current study which may have reduced power to detect between-group differences. The wound fluid collection device used herein was modified from previous studies (17, 18) to increase participant comfort. However, participants did not follow instructions to limit use of the wounded arm, which resulted in fluid leakage and missing data which precluded calculating AUC. Nevertheless, data from our previous study [18] did demonstrate differences in the wound fluid response between placebo and the treatment group. Therefore, the supplementation seems to

both favorably affect skin barrier restoration and, based on our prior work, local cytokine responses to short-term sleep restriction.

Study findings highlight the efficacy of nutritional interventions to support the innate immune system during and after sleep restriction and potentially other immune-degrading stressors. Findings from other studies support that contention. For example, collagen deposition was higher in healthy older adults who consumed a mixture of arginine (7 g), β-hydroxy-β-methylbutyrate (HMB, 3 g) and glutamine (14 g) versus placebo twice daily for 14 days (31). Further, higher collagen deposition at an experimental wound site and increased peripheral blood lymphocyte mitogenesis was observed in healthy younger (~30 years) and older adults (~70 years) in response to 30 $g \cdot d^{-1}$ of supplemental arginine compared to placebo (57, 58). Taken together, these data suggest that supplementing with higher protein diets and/or specific amino acids is beneficial for skin barrier restoration in healthy adults.

Other component(s) of the nutrient intervention used in this study may have also contributed to improved skin barrier restoration. In support, vitamin C concentrations status was below the normal reference range (22.7 – 114 $\mu mol \cdot L^{-1}$) (59) at baseline and at the beginning of the nutrition intervention period, but was within the normal reference range following the nutrition intervention period and demonstrated a tendency for a correlation with improvements in skin barrier restoration. Additionally, dietary intake of zinc during the five day post live-in period during the PLA trial was below the recommended daily intake of 11 mg (60). Given the established roles of zinc in promoting wound repair, the supplemental zinc provided during and after the sleep restriction in the NUT trial may have, along with vitamin C, also contributed to improvements in skin barrier restoration (61).

Biomarkers of immune function and stress (i.e., blood cytokines, tear SIgA, blood GH and cortisol) were unremarkable. Circulating markers of immune function were not affected by sleep restriction or the nutrition intervention. Further, while we observed changes in cortisol and GH concentrations with sleep restriction, we did not detect any treatment effects. These observations are consistent with our prior work (17) and other reports (3, 62-67) indicating that these biomarkers may not be as sensitive as local immune responses to external stressors (56, 68). This suggests that the shorter healing time observed in response to the nutrition intervention does not seem to be driven by changes in these stress/inflammatory biomarkers.

Monocyte recruitment to the wound site increased from 24-h to 48-h post-blistering with a subsequent decline in density (i.e., representing differentiation of cell populations), which was expected (69). The higher expression of HLADR monoclonal antibodies in cells recruited at the wound site during PLA compared to NUT is counter to what has been observed in previous sleep deprivation studies (70). However, our assumption that $CD45^{+}/CD14^{-}/CD16^{+}$ are predominantly composed of neutrophils (as opposed to migrating monocytes), based on the proportion observed at 24-h, explains this increase. Monocytes are recruited along with neutrophils immediately post-injury (71), and impaired neutrophil infiltration has long been purported to correlate with poor wound closure in both animal and human models (72, 73). Thus, our data suggest that the nutrition intervention improved wound healing at least partially through preferential recruitment of innate immune cells.

Several limitations warrant discussion. The first is the study design. It was not feasible to randomize treatment order for our study population (i.e., military personnel) to accommodate the >1 month washout period that is required due to nutrient content of the treatment beverage. To minimize potential bias, the following actions were taken: 1) volunteers were not informed of the

non-randomized design; the consent form and oral participant briefing did not emphasize dietary protein differences in the context of the study hypothesis or study design; and, main outcomes were objectively measured, thereby reducing the possibility of researcher bias. Most importantly, subjective perceptions of tiredness and fatigue were not different before or at the end of each treatment. The second limitation was the leakage of autologous fluid from the collection wells, which precluded assessing cytokine responses during the proliferative phase of the wound healing cascade and calculating AUC for that response. Low fluid volume also forced us to prioritize identifying cellular populations of interest mobilized in the wound fluid regardless of activation status. While cells mobilized in the blister sit are likely to be activated, future studies should aim to assess their activation status and effector profiles. Further, the design of the collection template will be modified in future studies to address well leakage, and more effective methods of immobilizing the arm will be implemented to better retain autologous fluid during the follow-up period. A third limitation was that protein intake was ~0.3 g·kg body weight^{-1}·day^{-1} higher than prescribed in the 5 days following PLA. However, the average protein intake from the total intervention period (live-in and post live-in period combined) was still significantly higher for NUT compared to PLA. An additional limitation was that females represented only 10% of the participants, and future studies should include a higher proportion of females due to sex differences in immune function (74). Lastly, there is some indication that long-term arginine supplementation may pose safety risks (75, 76), which should be considered when identifying possible intervention implementation opportunities.

CONCLUSION

Study findings extend our previous work, and indicate that an intervention including protein intake at almost double the RDA (or at the higher end of the MDRIs) in combination with a multi-nutrient beverage containing arginine, glutamine, zinc, vitamin C, vitamin D and omega-3

fatty acids mitigates delays in wound closure consequent to sleep restriction. These findings have practical implications when adequate sleep is not feasible (e.g., military personnel in training or combat, emergency service personnel or ultra-endurance athletic competitions), given that the potential for infection is heightened when the skin barrier is perturbed. Further research is necessary to determine whether findings persist when acute sleep restriction is combined with other physical and/or emotional stressors and to determine whether the effect of the nutrition intervention for mitigating delays in wound closure reduces infection risk in response to acute sleep restriction.

Conflict of Interest: *The authors declare that they have no competing interests*

Disclaimers

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or reflecting the views of the US Army or the Department of Defense. Any citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement of approval of the products or services of these organizations. Study funded by the US Army Medical Research and Materiel Command.

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T.S. conceived and designed the study, collected the data, conducted the analyses and drafted the manuscript and is responsible for the final content. M.W. collected the data and assisted with the data analyses related to immune function and skin barrier restoration. C.W. and H.F. coordinated recruitment, collected the data and assisted with the data analyses related to immune function, dietary intake and/or activity. K.H., C.S. and W.N. assisted in study design and contributed to data collection. C.C. contributed to data collection and provided technical support related to flow cytometry, while E.C. and G.S. assisted with analysis and interpretation of flow cytometry data and revised the article of important intellectual content. N.W. provided technical input related to tear collection and revised the article for important intellectual content. P.K. contributed to study design, contributed to data collection, assisted with data interpretation and revised the article for important intellectual content. All authors have read and approved the final version.

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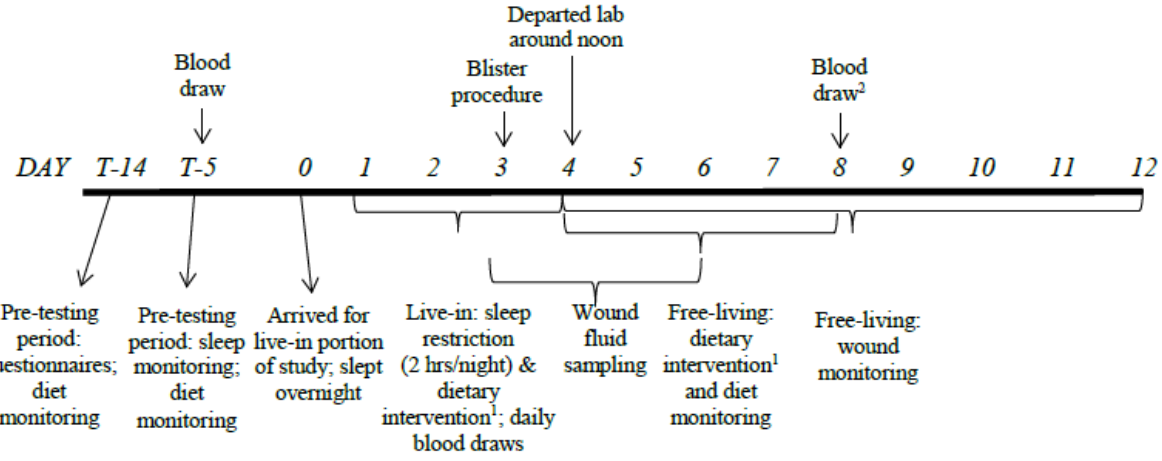

Fig. 1. Timeline of activities during each study phase.

[1]Dietary intervention consisted of 0.9 g·kg^{-1} body weight^{-1}·d^{-1} plus placebo beverage (PLA) or 1.5 g·kg^{-1} body weight^{-1}·d^{-1} plus multi-nutrient beverage (NUT).
[2]NUT only, to access micronutrient status before and after the nutrition intervention.

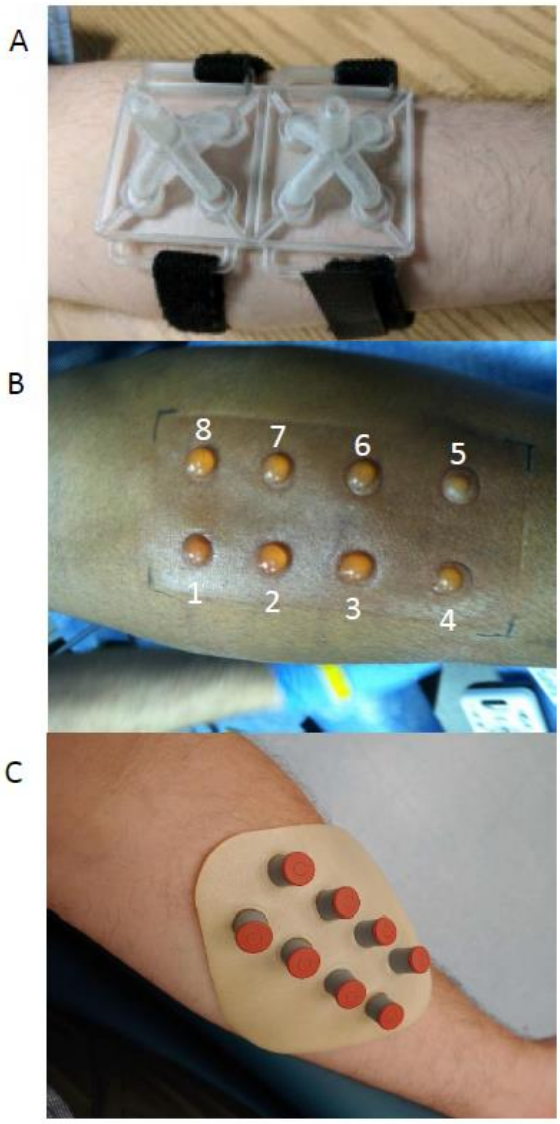

Fig. 2. Photographs of the suction blister template (A), the subsequent blisters (B), and the wound fluid collection template (C). Each blister was assigned a number to annotate location on the arm.

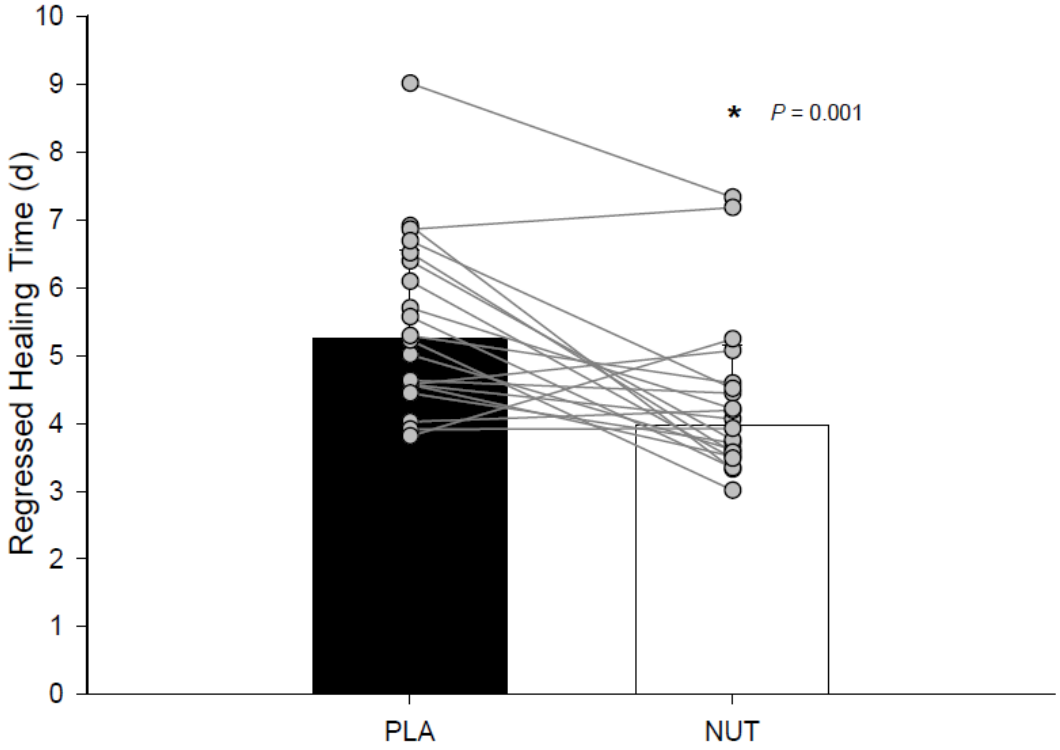

Fig. 3. Skin barrier restoration in healthy adults who underwent 72-h sleep restriction with (NUT) and without (PLA) nutrition intervention (n = 20).

Bars represent mean data for PLA and NUT with individual data shown with solid lines.

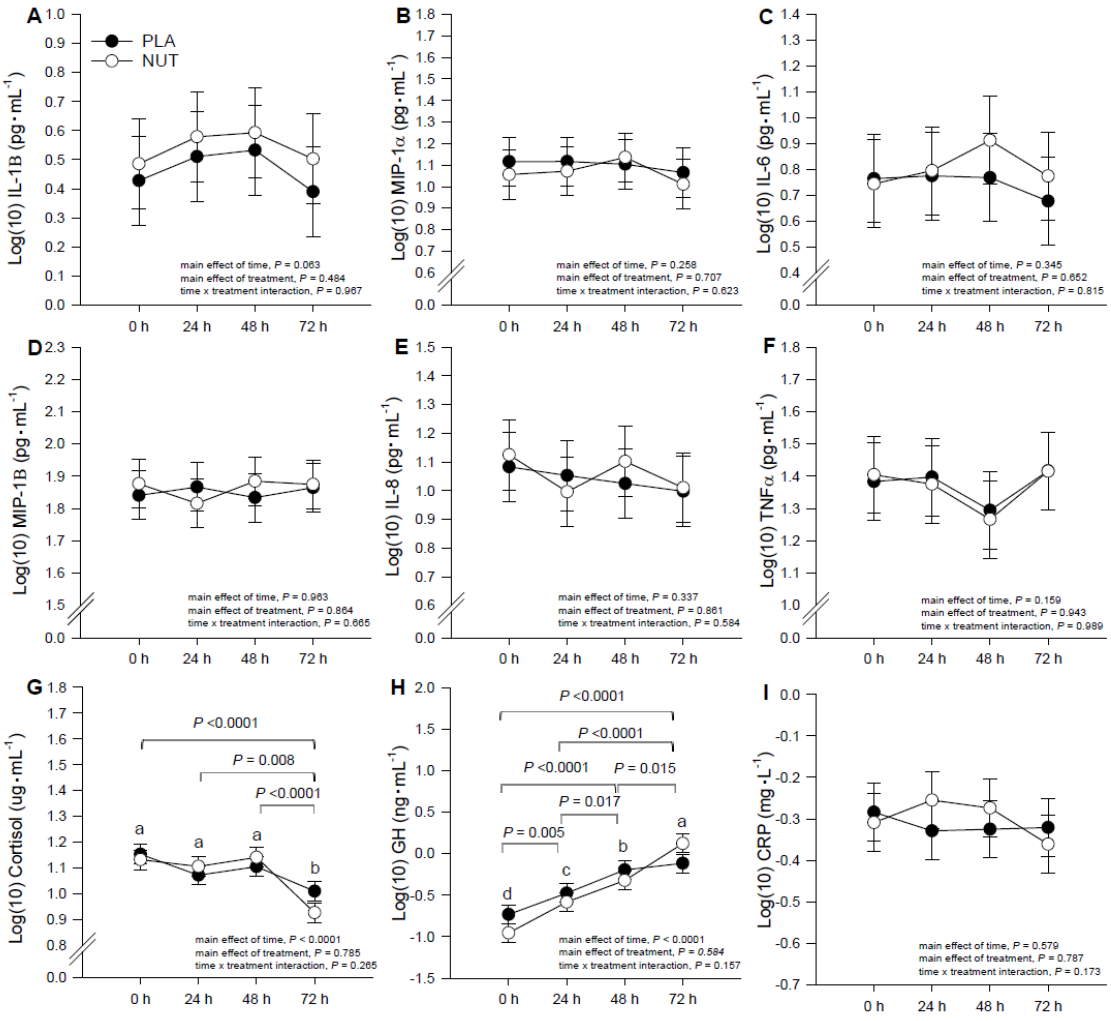

Fig. 4. Serum concentrations of serum C-reactive protein (CRP), growth hormone (GH), cortisol, and cytokines in healthy adults who underwent 72-h sleep restriction with (NUT) and without (PLA) nutrition intervention (n = 20).

Values are estimated marginal means ± SEM.
No time-by-treatment interactions were significant ($P \geq 0.05$); therefore, the interaction term was dropped and the model was re-run with main effects for time and treatment. Reported P-values for main effects of time and treatment reflect the final model that did not include the interaction term. Labeled timepoint means (treatments combined) without a common letter differ, $P < 0.05$ (t-test with Bonferroni correction).

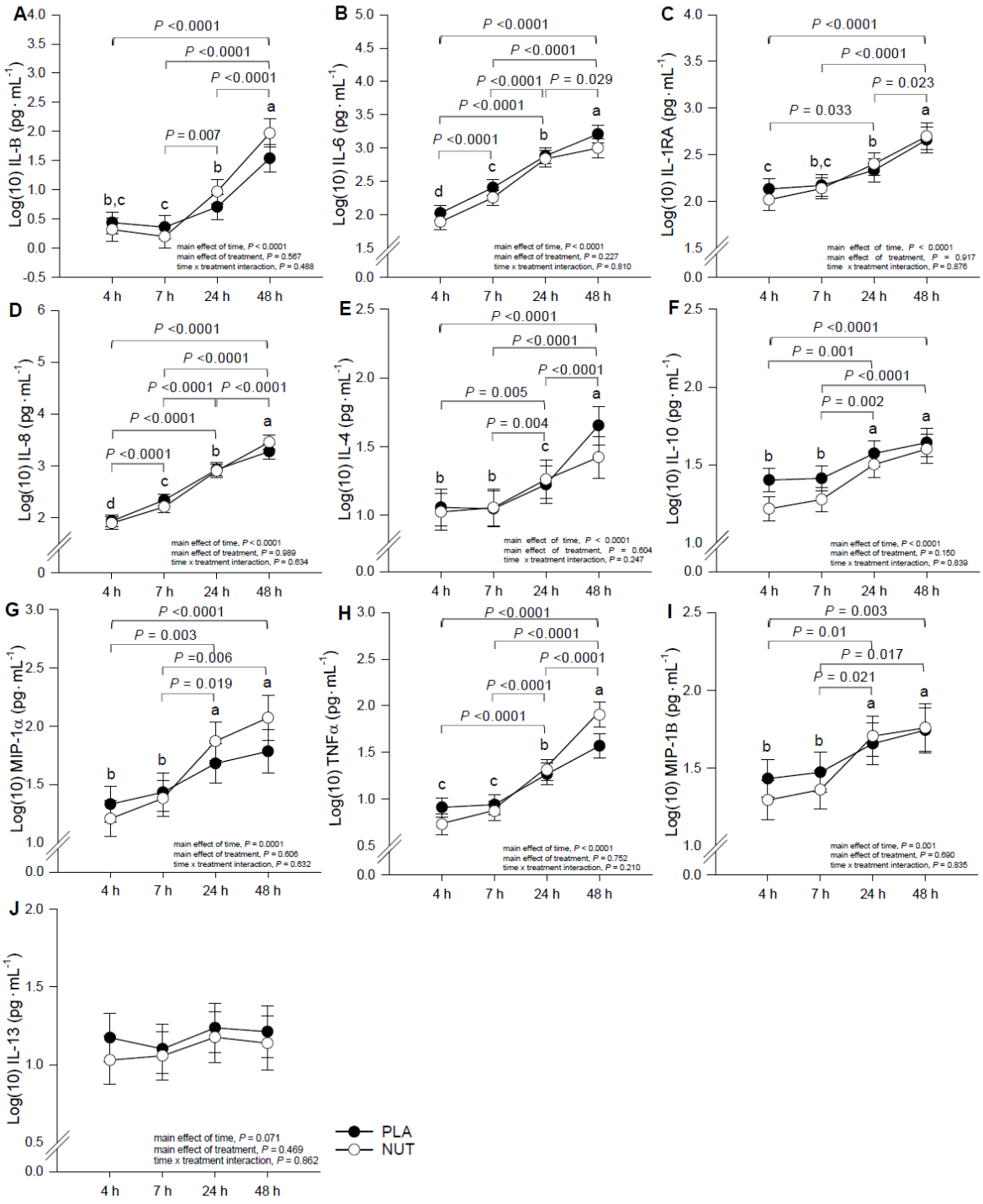

Fig. 5. Cytokine concentrations of wound exudate in healthy adults who underwent 72-h sleep restriction with (NUT) and without (PLA) nutrition intervention (n = 16).

Data points represent estimated marginal means ± SEM (n = 20);
MIP-1α: macrophage inflammatory protein-1α. MIP-1β: macrophage inflammatory protein-1β.

No time-by-treatment interactions were significant ($P \geq 0.05$); therefore, the interaction term was dropped and the model was re-run with main effects for time and treatment. Reported P-values for main effects of time and treatment reflect the final model that did not include the interaction term. Labeled timepoint means (treatments combined) without a common letter differ, $P < 0.05$ (t-test with Bonferroni correction).

Table 1. Baseline Characteristics for Healthy Adults[1]

Variables	
Age (years)	19.7 ± 2.30
Gender	
Male	18
Female	2
Race	
Hispanic/Latino	7
Not Hispanic/Latino	13
Ethnicity	
Caucasian	11
Black or African American	5
Other	4
BMI[2], kg·m-[2]	25.1 ± 2.80
Body Fat, %	23.0 ± 6.30
MEQ, total score	56.9 ± 5.30
PSQI, total score	2.70 ± 1.70
Sleep[3], h·(night^{-1})	8.11 ± 0.61
PSS, total score	29.2 ± 3.60
Serum Vitamin C, μmol·L^{-1}	20.8 ± 8.11
Serum 25-hydroxyvitamin D, ng·ml^{-1}	24.3 ± 8.34

[1]Values are mean ± SD, n = 20 or frequency
[2]BMI = Body mass index; MEQ = Morningness Eveningness Questionnaire; PSS = Perceived Stress Scale; PSQI = Pittsburgh Sleep Quality Index
[3]Hours of sleep per actigraphy monitoring

Table 2: Diet characteristics of healthy adults who underwent 72-h sleep restriction without (PLA) and with (NUT) multi-nutrient beverage[1]

	Time			*P*-values					
	Pre-study	Live-in	Post-study	PLA vs NUT pre-study	PLA vs NUT live-in	PLA vs NUT post-study	Time	Treatment	Time x Treatment
Energy, kcals·d^{-1} (PLA)	2500 ± 97.1	2790 ± 97.1	2400 ± 183	---	---	---	0.027	0.574	0.581
Energy, kcals·d^{-1} (NUT)	2670 ± 177	2750 ± 108	2420 ± 129						
Protein, g·d^{-1} (PLA)	70.1 ± 2.40^{b}	68.7 ± 2.49^{b}	93.9 ± 9.60^{a}	<0.0001	<0.0001	0.145	0.064	<0.0001	0.001
Protein, g·d^{-1} (NUT)	126 ± 8.98	106 ± 4.09	107 ± 6.24						
Protein, g·kg^{-1} body weight^{-1}·d^{-1} (PLA)	0.933 ± 0.009	0.915 ± 0.013	1.25 ± 0.127	<0.0001	<0.0001	0.090	0.088	<0.0001	0.001
Protein, g·kg^{-1} body weight^{-1}·d^{-1} (NUT)	1.70 ± 0.141	1.41 ± 0.019	1.46 ± 0.106						
Protein, % of total energy (PLA)	11.3 ± 0.194^{b}	9.88 ± 0.139^{c}	15.1 ± 0.833^{a}	<0.0001	<0.0001	0.001	<0.0001	<0.0001	<0.0001
Protein, % of total energy (NUT)	19.0 ± 0.554^{a}	15.5 ± 0.239^{b}	17.9 ± 0.720^{a}						
CHO[2], g ·d^{-1} (PLA)	354 ± 15.6	439 ± 15.5	313 ± 17.5	---	---	---	<0.0001	<0.0001	0.304
CHO[2], g ·d^{-1} (NUT)	297 ± 18.0	344 ± 16.8	246 ± 17.1						
CHO[2], % of total energy (PLA)	56.6 ± 0.648	63.1 ± 0.531	54.1 ± 2.14	---	---	---	<0.0001	<0.0001	0.496
CHO[2], % of total energy (NUT)	45.3 ± 1.53	49.7 ± 0.855	40.4 ± 1.59						
Fat, g·d^{-1} (PLA)	91.0 ± 3.32	89.5 ± 3.78	86.4 ± 10.4	---	---	---	0.079	0.169	0.125
Fat, g·d^{-1} (NUT)	110 ± 10.5	85.2 ± 3.87	90.1 ± 6.55						
Fat, % of total energy (PLA)	32.9 ± 0.477	28.8 ± 0.515	31.0 ± 1.47	---	---	---	<0.0001	0.033	0.066
Fat, % of total energy (NUT)	36.3 ± 1.48	27.9 ± 0.695	33.2 ± 1.22						
Arginine, g·d^{-1} (PLA)	4.37 ± 0.142^{a}	3.01 ± 0.140^{b}	3.05 ± 0.484^{b}	0.158	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Arginine, g·d^{-1} (NUT)	3.76 ± 0.439^{b}	23.0 ± 1.42^{a}	25.1 ± 1.53^{a}						
Glutamine, g·d^{-1} (PLA)	11.0 ± 0.259	11.9 ± 0.446	27.0 ± 12.4	---	---	---	0.102	0.001	0.543
Glutamine, g·d^{-1} (NUT)	28.5 ± 10.9	42.6 ± 2.07	44.4 ± 2.59						
Vit A, IU·d^{-1} (PLA)	3500 ± 159^{a}	2540 ± 98.0^{b}	5370 ± 1235^{a,b}	0.006	<0.0001	0.875	0.001	0.106	0.023
Vit A, IU·d^{-1} (NUT)	6740 ± 1031^{a}	2130 ± 86.1^{b}	5590 ± 821^{a}						
Vit C, mg·d^{-1} (PLA)	123 ± 2.73^{a}	115 ± 4.43^{a}	69.5 ± 0.61^{b}	0.164	<0.0001	0.018	0.058	0.001	0.033
Vit C, mg·d^{-1} (NUT)	102 ± 14.1^{b}	478 ± 5.48^{a}	805 ± 282^{a,b}						

Vit D, IU·d^{-1} (PLA)	307 ± 23.7^{a}	245 ± 17.0^{b}	97.7 ± 21.8^{c}	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Vit D, IU·d^{-1} (NUT)	169 ± 24.0^{b}	1210 ± 28.6^{a}	1090 ± 63.6^{a}						
Zinc, mg·d^{-1} (PLA)	11.3 ± 0.501^{a}	10.7 ± 0.333^{a}	7.69 ± 1.01^{b}	0.052	<0.0001	0.002	0.008	<0.0001	0.003
Zinc, mg·d^{-1} (NUT)	9.47 ± 0.944^{b}	37.8 ± 0.396^{a}	47.3 ± 11.1^{a}						
Omega 3 g·d^{-1} (PLA)	0.959 ± 0.039	1.21 ± 0.068	0.955 ± 0.163	---	---	---	0.056	0.003	0.060
Omega 3 g·d^{-1} (NUT)	1.04 ± 0.118	1.98 ± 0.054	2.69 ± 0.698						

[1]Values are estimated marginal means ± SEM, n = 20

[2]CHO, carbohydrate. PLA, 72-h Sleep Restriction with placebo. NUT, 72-h Sleep Restriction with nutrition intervention

When the interaction was significant, labeled means within a row without a common letter differ, P<0.05 (t-test with Bonferroni correction). When the time by treatment interaction term was not significant, the interaction term was dropped and the model was re-run with main effects for time and treatment. In these cases, reported P-values for main effects of time and treatment reflect the final model that did not include the interaction term, and dashes indicate that no post hoc testing to detect within- or between-treatment comparisons was conducted.