Impact of sleep restriction on local immune response and skin barrier restoration with and without ‘multi-nutrient’ nutrition intervention

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Authors’ contributions
Dr T. Smith conceived of and designed the study, collected the data, conducted the analyses and drafted the manuscript. Mr. Wilson collected the data and assisted in the data analyses related to immune function and skin barrier restoration. Dr. Karl contributed to study design and revised the article for important intellectual content. Drs. Heaton, C Smith and Cooper assisted in study design and contributed to data collection. Dr. Orr was responsible for data collection and analyses related to flow cytometry. Ms. Haskell and O’Connor were responsible for data collection related to dietary intake. Drs Montain and Young contributed to study conception and design and revised the article for important intellectual content. All authors reviewed, edited, and made important intellectual contributions to the manuscript.

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Systemic immune function is impaired by sleep restriction. However, the impact of sleep restriction on local immune responses, and to what extent any impairment can be mitigated by nutritional supplementation is unknown. We assessed the effect of 72-h sleep restriction (2-h nightly sleep) on local immune function and skin barrier restoration of an experimental wound, and determined the influence of habitual protein intake (1.5 g·kg\(^{-1} \cdot d^{-1}\)) supplemented with arginine, glutamine, zinc sulfate, vitamin C, vitamin D3 and omega-3 fatty acids compared to lower protein intake (0.8 g·kg\(^{-1} \cdot d^{-1}\)) without supplemental nutrients on these outcomes. Wounds were created in healthy adults by removing the top layer of \(\leq 8\) forearm blisters induced via suction, after adequate sleep (AS) or 48-h of a 72-h sleep restriction period (SR; 2-h nightly sleep). A subset of participants undergoing sleep restriction received supplemental nutrients during and after sleep restriction (SR+). Wound fluid was serially sampled 48-h post-blasting to assess local cytokine responses. The IL-8 response of wound fluid was higher for AS compared to SR (area-under-the-curve (log\(_{10}\)), 5.1±0.2 and 4.9±0.2 pg·mL\(^{-1}\), respectively (P=0.03); and, both IL-6 and IL-8 concentrations were higher for SR+ compared to SR (p<0.0001), suggestive of a potentially enhanced early wound healing response. Skin barrier recovery was shorter for AS (4.2 ± 0.9 days) compared to SR (5.0 ± 0.9 days) (P=0.02), but did not differ between SR and SR+ (P=0.18). Relatively modest sleep disruption delays wound healing. Supplemental nutrition may mitigate some decrements in local immune responses, without detectable effects on wound healing rate.

**Keywords:** sleep deprivation; cytokines; immune function; wound healing; skin barrier recovery; stress; Army; military personnel; first responders

**New and Noteworthy**

The data herein characterizes immune function in response to sleep restriction in healthy volunteers with and without nutrition supplementation. We used a unique skin wound model to
show that sleep restriction delays skin barrier recovery, and nutrition supplementation attenuates
decrements in local immune responses produced by sleep restriction. These findings support the
beneficial effects of adequate sleep on immune function. Additional studies are necessary to
categorize practical implications for populations where sleep restriction is unavoidable.
Immune responsiveness is degraded by short-term sleep restriction. The effect is mediated by hypothalamic-pituitary-adrenal axis and sympathetic nervous system activation (26), and characterized in part by decrements in natural killer cell activity and interleukin-2 production and increased levels of circulating proinflammatory cytokines (12, 27, 58). Collectively these effects are thought to increase risk of illness and infection (12, 43), and impair wound healing. For example, the risk of acquiring the ‘common cold’ was approximately 4-fold higher in volunteers who slept < 6 h per night, compared to those who sleep more than 7-h per night, for seven days (12, 43); and, 42-h total sleep deprivation delayed initial recovery of the skin barrier following an experimental wound. As such, developing strategies for maintaining immune function is of interest to populations in which short-term sleep restriction is sometimes unavoidable, such as military and emergency service personnel.

Optimal immune function is dependent on nutrient availability and underlying nutritional status (39, 54). Clinical nutrition support guidelines for adults recommend enteral formulations containing arginine, glutamine, omega-3 fatty acids, and antioxidants for immune-enhancement and faster recoveries in patients undergoing major elective surgery (35, 37, 39, 54). For example, vitamin C plays an important role in collagen synthesis, fibroblast proliferation, capillary formation and neutrophil activity (53) while omega-3 fatty acids enhance T-cell and natural killer cell activity, and have been shown to reduce systemic inflammation (11, 41). Further, studies indicate that certain nutrients improve wound healing indices in healthy adults (3, 32, 61). For example, arginine contributes to collagen deposition and cellular growth, and impacts microcirculation by increasing the production of nitric oxide (10, 39, 61), while glutamine stimulates the proliferation of fibroblasts, subsequently contributing to wound closure (39). The efficacy of nutrient interventions for modulating immune function and promoting healing in
healthy individuals who are immune-compromised consequent to physical or cognitive stressors (e.g., sleep restriction) has received less attention. The suction blister model is a useful tool for studying immune responsiveness of populations exposed to a variety of stressors and the efficacy of countermeasures to promote or enhance recovery. Traditionally, circulating blood-derived markers of immune function have been assessed to study the systemic immune response (12, 27, 58), but these markers do not fully characterize functional status (e.g., the ability to heal from a wound or defend against an infection) (20). In contrast, wound healing models directly assess functional status of the innate immune system (i.e., the ability to heal from a wound), and can also provide insight into the local pro-inflammatory response and tissue remodeling processes. The suction blister model is a wound healing model that allows study of the functional immune response to include immune response at a wound site along with skin barrier restoration as a proxy measure of wound healing rate. Our group has shown that this method is sufficiently reliable for assessing skin barrier restoration and local immune responsiveness of experimental skin wounds (50) (i.e., strong correlations were observed between the left and right arm in terms of skin barrier restoration rate and local cytokine response). Further, the method has been used in humans to study how stress affects in-vivo immune responsiveness (20, 30, 45). For example, skin barrier restoration was delayed by approximately one day following a 30-min adverse social interaction (i.e., verbal disagreement), compared to a 30-min positive social interaction, with their spouse (30); and, college examination stress delayed suction blister wound healing time by approximately two days (45). In addition to demonstrating decrements in the immune response, delayed wound closure has practical implications, i.e., the potential for infection is heightened while the skin
barrier is perturbed. This is relevant for military trainees, wherein cellulitis and purulent skin abscesses are a common problem (29).

The primary aims of this study were two-fold. We first sought to demonstrate that the suction blister wound model is sensitive enough to detect decrements in local immune response and skin barrier restoration rate in response to a stress model (i.e., 72-h sleep restriction with 2-h nightly sleep in a laboratory environment), by examining effects of the stress model on skin barrier restoration. After successfully demonstrating acceptable sensitivity, we sought to determine if dietary supplementation with arginine, glutamine, vitamin C, vitamin D, zinc and omega-3 fatty acids could mitigate decrements in local immune response and skin barrier restoration. We hypothesized that immune responses would be degraded, and skin barrier recovery would be delayed in participants following sleep restriction compared to free-living participants who were adequately rested. We further hypothesized that immune function would be preserved and skin barrier recovery would be shorter in participants who consumed 1.5 g protein per kg body weight (i.e., the higher end of the military dietary reference intake (MDRI), which was consistent with participants’ habitual protein intake) and a twice-daily, multi-nutrient beverage during and after sleep restriction compared to participants who received a placebo beverage and 0.8 g protein per kg body weight (i.e., the low end of the MDRI).

MATERIALS AND METHODS

Study Design

This was a two-phase study. Phase 1 determined the effect of sleep restriction and controlled living conditions (i.e., residing in the laboratory) on local immune responses and skin barrier restoration. Phase 2 determined the effect of a nutrition intervention on local immune response and skin barrier restoration in response to sleep restriction under controlled living.
conditions. In phase 1, the impact of short-term sleep restriction (i.e., ~72 hours of sleep restriction with 2-h sleep per night in a laboratory environment) on skin barrier restoration and immune response at the wound site was assessed by comparing ree-living participants with adequate sleep (AS) to a group of sleep restricted participants who resided in the laboratory during the sleep restriction period (SR) (Figure 1). In phase 2, we determined if a diet providing 1.5 g protein per kg body weight combined with a multi-nutrient supplement during and after sleep restriction (SR+) attenuated the decrements in local immune function and skin barrier restoration observed in response to sleep restriction when compared with a diet providing 0.8 g protein per kg body weight combined with a placebo beverage (SR) (Figure 1). Dietary protein levels were selected to represent the lower and higher ends of the MDRIs (0.8-1.6 g protein per kg body weight per day)(56), and the higher-level of 1.5 g protein per kg body weight per day was consistent with participants’ reported habitual protein intakes. Blisters were applied to all three study groups (AS, SR and SR+), and the main measures of immune function included skin barrier restoration (measured by skin vapor permeability) and wound inflammatory responses. Participants

Participants were military and civilian personnel assigned to Natick Soldier Systems Center, Natick, MA. Eighty-five percent (n = 56) of the 66 participants who began the study completed data collection and were included in the data analyses (AS, n = 16; SR, n = 20; and, SR, n = 20). One participant withdrew prior to study participation due to scheduling conflicts, seven volunteers withdrew during baseline testing (i.e., n = 3 due to relocation from the geographical area; and, n = 4 due to non-compliance with the sleep requirements leading up to the sleep restriction period), and three participants left the study during the sleep restriction
period (i.e., SR, n = 2 due to gastrointestinal virus or migraine; and, SR+, n = 1 due to inability to stay awake).

Data collection occurred from September 2012 to May 2016 at the U.S. Army Research Institute of Environmental Medicine (Natick, MA). Each volunteer gave their written, informed consent after an oral explanation of the study. Individuals were included if they were between the ages of 19 and 35 years, were generally healthy and not taking medications (including nonsteroidal anti-inflammatory drugs and aspirin), were not pregnant or lactating, had no history of psychiatric disorder requiring hospitalization or psychiatric medication usage, and slept between 7 and 9 hours per night at least five days per week. 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. The Clinicaltrials.gov identifier is NCT02053506.

Research procedures applicable to all experimental groups (AS, SR & SR+)

Assessment of General Sleep Patterns

Participants confirmed that they regularly slept 7-9 hours per night prior to the baseline testing period. General sleep patterns were assessed during the baseline testing period via the Morningness/Eveningness questionnaire, actigraphy, and a paper-and-pencil sleep diary. The Morningness/Eveningness questionnaire (23) is a 19-item questionnaire that assesses respondent’s circadian preference, sleep-wake pattern for activity, and morning and evening alertness; and was used as an initial screener wherein participants needed to score between 31 and 69 to remain in the study, thus avoiding extremes in “morningness” or “eveningness”.
Participants wore an actigraphy monitor (Actical, Philips Respironics, Murrysville, Pennsylvania or an equivalent) for five days prior to the blister induction (AS) or live-in portion of the study (SR and SR+) to verify that they 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.

Assessment of Life Stressors

The Perceived Stress Scale (13) was administered to all participants either within a week of the blister induction (AS) or upon arriving to the lab for the live-in portion of the study (SR and SR+) to assess life stressors in the previous month. This scale is a reliable and valid 14-item, widely used self-report measure of perceived stress, wherein respondents rate the stressfulness of their life during the previous month. The items are answered on a 0 (never) to 4 (very) scale, with higher sum scores indicating greater perceived stress.

Anthropometrics

Standing height was measured at baseline, in duplicate using an anthropometer (Seritex, Inc., Carlstadt, NJ or similar). Body weight was measured in shorts, t-shirt, stocking feet) at baseline and either the morning of the blister induction (AS) or each day of the live-in portion of the study (SR and SR+) using a calibrated electronic scale (Tanita WB-110A Class III, Tokyo, Japan).

Suction Blister Induction and Fluid Sampling

Venous blood was drawn from the forearm on the morning of the blister induction, and ~3.0 mL of serum was used to prepare an autologous fluid mixture to be used in the suction blister model (30% serum and 70% Hanks (+) buffer solution). CRP was assessed from serum and analyzed in duplicate using Multiplex bead based on Luminex® technology.
Suction blisters were induced according to previously described methods (50). Briefly, a vacuum pressure was applied to a polycarbonate template on the forearm to form 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, which acts as a soluble chemotactic substance (62), was syringed into the polycarbonate wells. The concentration of inflammatory cytokines (IL-1β, IL-6, IL-8, TNF-α, MIP-1α and MIP-1β) was assessed by removing fluid from distinct wells at 4-h (AS, SR and SR+), 7-h (AS, SR and SR+), 24-h (AS, SR and SR+), and 48-h (SR and SR+) following blister formation.

**Transepidermal Water Loss (TEWL) to Assess Skin Barrier Restoration**

The time to skin barrier restoration was assessed by measuring TEWL from individual blisters using the VapoMeter (Delfin Technologies Inc., Stamford, CT). Beginning ~24-h after blister formation, TEWL was measured twice each morning, from the lower four wound sites and an adjacent, non-wounded, control site, and the paired measurements were averaged. If values were not within 10% of each other, a third measurement was taken and the two closest values were averaged. The TEWL measurements from wound number six were used to assess skin barrier restoration, since the majority of participants developed a blister at this location (i.e., all participants in AS and SR, and 18 of 20 participants in SR+) and our prior work indicated that blister size was consistent between participants at this site. A ‘Standard of Recovery’ was established using the TEWL values ~24 hours after blister induction (30):

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\text{[TEWL measurement from wound site} - \text{TEWL measurement from control site (both measured ~24 hrs after blister induction) x 0.10}
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The skin barrier was considered “restored” when a subsequent daily TEWL value (i.e., wound site measurement minus control site measurement) met or exceeded the ‘**Standard of Recovery**’.
‘Standard of Recovery’, were then exponentially regressed to better identify the precise moment of skin barrier restoration.

Additional Research Procedures Applicable Only to Sleep Restricted Participants (SR and SR+)

Participants underwent approximately 72 hours of sleep restriction with 2-h sleep per night in the laboratory to induce decrements in immune responsiveness and delay skin barrier restoration, and to identify if additional protein combined with a multi-nutrient beverage could mitigate these decrements (SR compared to SR+, Figure 1). Suction blisters were induced after 48-h of the sleep restriction protocol. The 72-h duration of sleep restriction with limited nightly sleep was selected based on the somewhat typical wake-restricted sleep that military personnel encounter during training (8) and combat missions ((34). The sleep-wake pattern in this study is also relevant to non-military emergency service personnel and medical interns, who may also encounter short-term scenarios where sleep restriction is unavoidable (4, 55, 59); and, endurance athletes who may self-impose sleep restriction during short-term, multi-day events (24, 33, 42).

Study participants arrived to the laboratory the day before the sleep restriction period began, and slept overnight at the laboratory. During the ~72 hour sleep restriction period, participants slept only 2 hours per night and engaged in a variety of activities to maintain wakefulness (e.g., exercise, video games, television, movies), similar to the activities that were performed by participants in the free-living group that received adequate sleep (AS).

Determination of Total Daily Energy Expenditure

Total Energy Expenditure (5) during the sleep restriction period was estimated to determine the level of energy intake required to maintain body weight for each participant during the nutrition intervention experiment. TEE was estimated from approximate time spent sleeping,
participating in miscellaneous activities (e.g., eating, watching TV, playing video games, personal care, moving about the dorm area, etc...), and exercise (46).

Physical Activity

The purpose of including mild to moderate physical activity during the sleep restriction period was 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 that participants performed during the sleep restriction period. Exercise consisted of treadmill walking, outdoor walking and cycle ergometry. The American College of Sports Medicine’s Metabolic Equations for steady state exercise conditions were used to estimate exercise workloads and subsequent energy expenditure (40). Trained study staff confirmed that all physical activity was performed at light intensity (self-reported using the 20-point Borg RPE scale (7).

Assessment of Dietary Intake

Intake of omega-3 fatty acid-rich foods, probiotics and other dietary supplements (including multi-vitamin/minerals) was assessed at baseline by questionnaire, along with oral antibiotic use; and, participants were asked to refrain from consuming these items for the duration of the study. 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 quantify intake of energy and macronutrients, as well as 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

During sleep restriction period (Figure 1): Participants consumed measured and provided diets 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 ~0.8 grams·kg\(^{-1}\) body weight·day\(^{-1}\), which is the low end of the MDRI (SR) or ~1.5 grams·kg\(^{-1}\) body weight·day\(^{-1}\), which is the higher end of the MDRI (SR+). The higher level of protein was chosen for the intervention diet based on general recommendations for immune-supporting diets, since proteins are a vital component of collagen synthesis (10, 37, 39, 54). 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 other food or beverages other than those provided.

Post-sleep restriction period (Days 4-8, Figure 1): upon leaving the lab on Day 4, participants were instructed to consume a protein-controlled (SR: ~0.8 grams·kg\(^{-1}\) body weight·day\(^{-1}\); SR+: 1.5 grams·kg\(^{-1}\) body weight·day\(^{-1}\)), ad libitum diet. 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.
The multi-nutrient beverage contained L-arginine (20 g·d⁻¹), L-glutamine (30 g·d⁻¹), omega-3 fatty acids (1 g·d⁻¹), zinc sulfate (24 mg·d⁻¹), vitamin D3 (800 IU·d⁻¹) and vitamin C (400 mg·d⁻¹). Content of the multi-nutrient beverage was based on formulas used in clinical settings which have shown benefits related to post-surgical infectious complications (9, 14, 15, 49, 51) and wound healing disorders (17). Nutrients were purchased from DSM Nutrition Products (Parsippany, NJ). The nutrient “pre-mix” (containing the arginine, glutamine, zinc, vitamin D and vitamin C) was added to an artificially sweetened, commercially available beverage powder using good manufacturing practices (4C Totally Light, Brooklyn, NY); and, the omega-3s (i.e., 500 mg docosahexaenoic acid, 300 mg eicosapentaenoic acid and 150 mg short-chain omega-3 fatty acids) were packaged separately. The placebo beverage was composed of the same commercially-available, artificially sweetened beverage powder (4C Totally Light, Brooklyn, NY) and 0.03 g of naringen and 0.004 g of quinine (both from Penta Manufacturing, Livingston, NJ) to impart a slightly bitter taste to match the taste of the ‘multi-nutrient’ beverage. The powders were stored in the refrigerator (omega-3 powder) or freezer (‘multi-nutrient’ and placebo beverage powders); and, were added to containers and reconstituted with water prior to consumption. 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.

Systemic Markers of Inflammation and Immune function
Whole blood was drawn from a forearm vein daily, upon waking, during the live-in portion of the study. Cortisol, growth hormone, CRP, and cytokines were assessed from serum. Cytokines and CRP were measured, in duplicate, using Multiplex bead based on Luminex® technology. Cortisol and growth hormone were measured using the Immulite immunoassay system (Siemens Healthcare, Erlangen, Germany). Vitamin C and 25-hydroxyvitamin D were measured from blood on the morning of day 1 before sleep restriction to determine background micronutrient status, using colorimetric (BioVision, San Francisco, CA) and enzyme linked immunosorbent assay kits (R&D Systems, Minneapolis, MN) respectively.

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. Mean and variance data from our prior work (50) were used for sample size calculations, and indicated that 20 participants were required to detect a 0.75 day (or 15%) difference in skin barrier restoration time between groups (α = 0.05, power = 0.80). Sample size calculations using 24-hr concentrations of IL-8, IL-6 and TNF-α from our prior work (50) indicated that ~20 participants were required to detect a ~40% difference in cytokine response (α = 0.05, power = 0.80).

Area-under-the curve (AUC) values were calculated for each participant using data obtained from autologous wound fluid sampled following blister formation (i.e., wound cytokines concentrations). Briefly, “Area under the curve with respect to the increase” (AUCi) (44) represents the total AUCi for all measurements with consideration for the time difference between each measurement and their distance from the baseline value. In the few cases (n = 3) when no autologous wound fluid was available at the designated time-point(s) due to leakage from the well(s), the group mean was substituted in place of the missing value to calculate AUCi.
In cases where values were either more than 3 SDs from the mean or below the limits of detection, either the group mean or zeros, respectively, were substituted to calculate AUCi.

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 (i.e., cytokine serum and wound concentrations, CRP and GH) were log transformed (log10). Repeated measures analysis of variance (ANOVA) was used to assess changes in body weight over time. Independent samples t-test was used to determine differences between AS and SR, and SR and SR+ for skin barrier restoration rate, cytokine concentrations from post-blister wound fluid (AUCs), and baseline measures (i.e., MEQ and PSS scores, CRP, 25-hydroxyvitamin D status, vitamin C status, and average sleep). The study was not powered to compare AS versus SR+, because this comparison was not of interest.

Additionally, linear mixed models with first order autoregressive covariance type was used to determine main effects of time and condition, and their interactive effects with regard to cytokine concentrations in blister wound fluid and serum, and GH, CRP and GH concentrations. When significant main effects or interactions were observed, all possible t-tests were conducted and the Bonferroni correction was used to control the familywise error rate. Results are presented as mean (± SD), unless otherwise noted. A two-tailed "p" value of 0.05 was considered statistically significant.

RESULTS

Baseline Measurements

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.
There were no significant differences in any baseline measures between AS and SR or SR and SR+, with the exception of higher 25-hydroxyvitamin D concentrations for SR compared to SR+ (Table 1).

**Effect of sleep restriction on local inflammation and skin barrier restoration (AS versus SR)**

Time to skin barrier restoration was significantly higher for SR (5.0 ± 0.9 days) compared to AS (4.2 ± 0.9 days, P = 0.02). The analysis was repeated without females, to ensure that the sex imbalance between groups wasn’t a confounder, and results were unchanged (AS, 4.1 ± 1.0, and SR, 5.0 ± 0.9, P=0.02). These results confirmed the usefulness of the suction blister model for detecting immune function decrements in response to sleep restriction and controlled living conditions. This finding provided rationale supporting Phase 2, to determine if a nutrition intervention could mitigate immune function decrements in response to sleep restriction under controlled living conditions. Cytokine values from 13% of the 288 wells (AS and SR combined) were excluded from calculations, since less than 70% of autologous serum added to the chambers immediately post-blistering was recovered from these wells at the follow-on time-points. Wound fluid concentrations of IL-6, IL-8, MIP-1α, MIP-1β and TNF-α significantly increased over time for both AS and SR. A group x time interaction was observed for IL-8 (p<0.0001), wherein the mean concentration was higher for SR compared to AS at 7-h (log$_{10}$ 2.6 ± 0.4 pg·mL$^{-1}$ and log$_{10}$ 2.3 ± 0.3 pg·mL$^{-1}$, respectively, P = 0.004); and, IL-8 concentration over the total sampling period (i.e., AUC$_{log10}$) was significantly higher for AS compared to SR (5.1 ± 0.2 pg·mL$^{-1}$ and 4.9 ± 0.2 pg·mL$^{-1}$, respectively, P = 0.03). No other significant between group differences were detected.

**Effect of a multi-nutrient beverage on local inflammation and skin barrier restoration in response to sleep restriction (SR versus SR+)**
Anthropometrics, Energy Expenditure & Dietary Intake

Average TEE for SR and SR+ was 2860 ± 410 kcals·d⁻¹ and 2820 ± 610 kcals·d⁻¹, respectively (P = 0.8), with EEE contributing 470 ± 170 kcals·d⁻¹ and 460 ± 200 kcals·d⁻¹ to TDEE, respectively (P = 0.9). Body weight was not significantly different over time within or between groups during the sleep restriction period (P = 0.5 and P = 0.6, respectively), indicating that participants were in energy balance. There were no differences in dietary intake between SR and SR+ prior to the 72-hr live-in periods (Table 2). Per the study design, dietary intake of protein (i.e., total grams, grams per kg body weight and percent of total energy intake), arginine, glutamine, omega-3 fatty acids, vitamin D, vitamin C and zinc were significantly higher for SR+, compared to SR, during and after the sleep restriction period (Table 2). Vitamin A was also significantly higher for SR+ compared to SR ([mean difference ± SE] 512 ± 91 IU, p < 0.0001), during the sleep restriction period, due to higher intake of cheese products in the prescribed study diet. Daily energy intake was [mean difference ± SE] 585 ± 146 kcals lower for SR compared to SR+ during the 5-d follow-up period (p<0.0001).

Multi-nutrient Beverage

Compliance with the beverage prescription during and after the sleep restriction period was 100% and 99.7%, respectively (i.e., one participant in SR+ reportedly forgot to consume the beverage on the afternoon of Day 7, thus consumed only half of the daily dose of nutrients provided by the beverage on that particular day).

Systemic Markers of Inflammation and Immune function

For both SR and SR+, serum GH concentrations on day 1 were significantly lower, and circulating cortisol concentrations were significantly higher, compared to all other time points. 

Figure 3. No within group changes over time were detected for CRP. A group x time
interaction (p<0.0001) was detected for cortisol which tended to be higher, or was significantly higher, in SR compared to SR+ on the morning of day 2 ([mean difference ± SEM] 2.1 ± 1.2 µg/dL and, P=0.07) and day 4 ([mean difference ± SEM] 4.7 ± 1.2 µg/dL, p<0.0001), respectively Figure 3.

There were no within group changes for SR and SR+ in terms of IL-1β, IL-6, IL-8, MIP-1α and MIP-1β serum concentrations; however, TNF-α serum concentration significantly declined from day 3 (log$_{10}$ 1.253 pg·mL$^{-1}$) to day 4 (log$_{10}$ 1.156 pg·mL$^{-1}$, P = 0.002) for SR.

Although there was a main group effect (p<0.05), wherein serum concentrations were higher for SR compared to SR+ with regard to IL-8 (Days 1-4, p < 0.0001), TNFα (Day 1 and Day 2, P = 0.05; and, Day 3, P = 0.03), and MIP-1β (Days 1-4, p < 0.0001), there were no significant group-by-time interactions for any of the measured serum cytokines.

**Immune response of autologous wound fluid**

Cytokine values from 10% of the 320 wells (SR and SR+ combined) were excluded from calculations, since less than 70% of autologous serum added to the chambers immediately post-blistering was recovered from these wells at the follow-on time-points. Autologous wound fluid concentrations of IL-1β, IL-6, IL-8, MIP-1α, MIP-1β and TNF-α significantly increased over time for both SR and SR+, with significant time x group differences (Figure 4). Additionally, AUC$_i$ concentrations were higher for SR+ compared to SR with regard to IL-6 (log$_{10}$ 5.7 ± 0.3 pg·mL$^{-1}$ and 5.3 ± 0.3 pg·mL$^{-1}$, respectively, p < 0.0001) and IL-8 (log$_{10}$ 6.3 ± 0.5 pg·mL$^{-1}$ and 5.6 ± 0.1 pg·mL$^{-1}$, respectively, p < 0.0001) (Figure 4). Results were not changed when baseline 25-hydroxyvitamin D concentrations, vitamin A intake during the sleep restriction period or energy intake during the 5-day recovery period were used as covariates.

**Skin Barrier Restoration**
Time to skin barrier restoration was not significantly different between SR (5.0 ± 0.9) and SR+ (4.6 ± 0.8 days), P = 0.18; and, results were unchanged when baseline 25-hydroxyvitamin D concentrations, vitamin A intake during the sleep restriction period or energy intake during the 5-day recovery period were used as covariates.

DISCUSSION

In this investigation we confirmed our initial hypothesis that skin barrier restoration was delayed for participants who underwent 72-h of sleep restriction with 2-h of sleep per night in a laboratory compared to participants who were adequately rested, with some degradation in cytokine response at the wound site during the initial phases of wound healing. Our second hypothesis was partially confirmed, wherein concentrations of pro-inflammatory cytokines at the wound site were higher during the initial phase of wound healing for participants who consumed habitual protein intake and a twice daily multi-nutrient beverage during and after 72-h of sleep restriction with 2-h sleep per night compared to participants who received a lower protein intake with placebo beverage. However, we were unable to detect differences in skin barrier recovery in response to the nutrition intervention.

The finding that sleep restriction in a laboratory environment delayed skin barrier recovery, compared to free-living, adequately rested participants, is consistent with studies that investigated the impact of chronic and acute psychological stress on wound healing (2, 21, 30, 31, 36). Further, Altemus et al. (2001) suggested that skin barrier function was perturbed after 42-h of total sleep deprivation, however, authors only measured skin barrier recovery within 3 hours following tape stripping (i.e., up to 75% recovery). Using the suction blister model, Kiecolt-Glaser et al. (2005) reported that skin barrier restoration was delayed by ~1 day following a 30-min adverse social interaction (i.e., verbal disagreement), compared to a 30-min positive social interaction, with their spouse. Roy et al. (2005) similarly reported that college
examination stress delayed suction blister wound healing time by ~2 days. Taken together, findings from the current study and past investigations confirm that stressors, including sleep restriction, ultimately delay healing of an experimental wound. That decrements in wound IL-8 concentration over the total sampling period in the present study were lower in participants who underwent sleep restriction in a laboratory compared to free-living participants who were adequately rested suggests that the delayed wound healing in response to sleep restriction in a laboratory may be attributed to perturbations in the inflammatory response during the critical early phases of wound healing, as has been previously suggested by studies of psychological stress (20, 30). However, decrements in the later phases of the wound healing cascade may also be responsible for delayed healing after sleep restriction which can be assessed in future trials by sampling wound fluid beyond 48-h post-blistering.

We did not detect any changes in circulating markers of immune function over the course of the sleep restriction period, compared to baseline concentrations, with the exception of a decline in serum TNF-α concentration from day 3 to day 4 of the sleep restriction period. Of note, circulating cytokines on the morning of “day 1” may not have been an accurate depiction of “baseline” concentrations given that participants spent the previous night in the laboratory (i.e., an unaccustomed environment) and were potentially anxious about the impending sleep restriction and related study activities. In support, the baseline concentrations of IL-1β and TNF-α in the current study were more than three-fold higher than the pre-sleep restriction values reported by Altemus et al (2001). Regardless, acute sleep loss, either partial or total, seems to produce differential results with regard to the peripheral inflammatory cytokine response with some studies showing an increased production of pro-inflammatory cytokines (2, 18, 22, 25, 28, 57, 58) and others reporting no change over time (1, 48, 52). Further, cytokine concentrations...
from peripheral blood are thought to provide a ‘snapshot’ of systemic immunity, but may not reflect local immune responses (20, 30).

We did not detect significant differences in skin barrier recovery in participants who consumed supplemental nutrients during and after the sleep restriction period compared to those who received a non-nutritive placebo. The failure of the nutrition intervention to affect skin barrier recovery was surprising, given the higher concentrations of pro-inflammatory cytokines at the wound sites during the initial phase of wound healing in participants who received the nutrition intervention relative to those who received the placebo. There are few studies that have tested the efficacy of nutrition interventions on wound healing outcomes in healthy adults. Williams et al. (2002) reported that collagen deposition, as measured by the content of hydroxyproline in a subcutaneously-placed catheter, 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. Two other studies supplemented the diets of healthy younger (~30 years) and older adults (~70 years) with 30 g/d of arginine and demonstrated higher collagen deposition at an experimental wound site and increased peripheral blood lymphocyte mitogenesis compared to placebo (3, 32). Those findings lead us to expect that the nutrition intervention would accelerate skin barrier restoration. However, another published human study that tested the efficacy of a nutrition intervention (i.e., 4 weeks of eicosapentaenoic/docosahexaenoic polyunsaturated fatty acid, PUFA, supplements) on skin barrier restoration did not detect a significant difference between the experimental and placebo group (38). Therefore, it is possible that the nutritional intervention used in this study lacked the effect magnitude to allow detection of improved healing time.
We observed that the nutrition intervention group experienced higher IL-6, IL-8 and MIP-1β concentrations at the wound site during healing, compared to the placebo group, which potentially indicates an enhanced response during the early phases of wound healing. These findings are consistent with Martinez et al. (2004) and McDaniel et al. (2008) who also reported higher cytokine expression at a wound site (i.e., IL-1β, with a trend for higher IL-6 and TNF-α, concentrations) within 24-h of blistering, with no significant differences in healing time in participants who were either supplemented with PUFA or placebo for 4 weeks. Although the functional significance of altered local cytokine concentrations in the present context remains unclear, increased expression of pro-inflammatory and chemotactic cytokines during the initial phase of wound healing is thought to be advantageous given their multifaceted roles that are expected to promote healing, e.g., enhancing phagocytosis, stimulating the migration of keratinocytes at the edges of the wound, promoting fibroblast chemotaxis and proliferation, and stimulating re-epithelialization, tissue remodeling and the formation of new blood vessels (16, 47, 60). Taken together, the somewhat equivocal findings in the current study suggest that additional research is warranted to elucidate the functional implications of enhancing local immune responses.

Contrary to expectations, we did not detect an increase in cortisol concentrations or decline in growth hormone over the course of the sleep restriction period, as has generally been demonstrated in the literature in response to acute sleep restriction (6, 19, 26, 48). Similar to the current study’s results, Altemus et al. (2001) reported no change in cortisol in response to 42-h of total sleep deprivation compared to baseline levels (6.1 ± 0.9 and 5.5 ± 0.9 µg/dL, respectively). Since normal variations in hormone concentrations (e.g., cortisol) occur across a 24-h period, it
would be advantageous to sample circulating levels at multiple time-points throughout the day to further characterize the response and also to measure catecholamines.

This study has limitations that should be considered in terms of data interpretation and planning of future trials. We cannot exclude the possibility that differences in housing conditions between AS and SR contributed to the observed differences between these groups. However, any stressors experienced during the free-living AS phase would be expected to bias results towards the null and living in the laboratory during the sleep restriction period was part of the stressor in Phase 1 of the study. Additionally, greater-than-expected variability between participants may have decreased our ability to detect differences between the sleep restriction groups in terms of skin barrier restoration. Additionally, both groups reportedly consumed ~1.5 g protein per kg body weight leading up to the sleep restriction period, which was consistent with the prescribed protein intake for SR+ but higher than the prescribed protein intake of SR, thus potentially confounding the results. Therefore, including a protein-controlled diet prior to the intervention to habituate liver enzymes to the prescribed protein intake is prudent. Lastly, while energy intake was different between the sleep restriction groups during the five day follow-up period, the estimated energy imbalance was < 100 kcals·d⁻¹ for three of the five days. Further, it’s unlikely that a mild energy deficit for the remaining two days affected wound healing (54) and results were unchanged when energy intake was included as a covariate in the analysis.

Despite these shortcomings, this study provides valuable insight into the local pro-inflammatory response and tissue remodeling processes, and nutritional interventions to support the innate immune system, during and after sleep restriction.

CONCLUSION

Herein, we demonstrate that the suction blister model is an effective model for testing the
immune response to stressors and for testing the efficacy of countermeasures to mitigate immune decrements (e.g., nutrition interventions) during stress. Using this model we showed that 72-h of sleep restriction with 2-h sleep per night in a laboratory delays skin barrier recovery, thus underscoring the importance of adequate sleep when feasible. However, when adequate sleep is not feasible (e.g., military personnel in training/combat, emergency service personnel, ultra-endurance athletic competitions), these findings suggest that maintaining protein intake at the higher end of the MDRIs in combination with a multi-nutrient beverage (i.e., containing arginine, glutamine, zinc, vitamin C, vitamin D and omega-3 fatty acids) may attenuate some decrements in local immune responses observed during sleep restriction relative to a lower protein intake without nutrient supplementation, albeit without affecting skin barrier recovery. Additional research is needed to elucidate the functional implications of this improved local immune response.

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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Reference List


<table>
<thead>
<tr>
<th>Baseline Data</th>
<th>Adequate Sleep (n = 16)</th>
<th>72-h Sleep Restriction (n = 20)</th>
<th>72-h Sleep Restriction with nutrition intervention (n = 20)</th>
<th>P-value AS vs SR</th>
<th>P-value SR vs SR+</th>
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<td>Age</td>
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<td>BMI (kg/m²)</td>
<td>25.7 ± 3.7</td>
<td>26.5 ± 3.8</td>
<td>26.2 ± 3.8</td>
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<td>MEQ (total score)¹</td>
<td>56.2 ± 6.4</td>
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<td>54.7 ± 4.2</td>
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<td>Sleep (hrs/night)²</td>
<td>7.99 ± 0.50</td>
<td>7.71 ± 0.67</td>
<td>7.64 ± 0.50</td>
<td>0.18</td>
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<td>PSS (total score)³</td>
<td>28.4 ± 3.1</td>
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<td>C-reactive protein log₁₀ (mg/L)</td>
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¹Morningness Eveningness Questionnaire
²Hours of sleep per actigraphy monitoring
³Perceived Stress Scale
Table 2: Diet characteristics of participants who underwent 72-h sleep restriction without (SR) and with (SR+) multi-nutrient beverage

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>72-h Sleep Restriction (SR)</th>
<th>72-h Sleep Restriction with nutrition intervention (SR+)</th>
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<td>Energy (kcal)</td>
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<td>Mean ± SD</td>
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<tr>
<td>Protein (g)</td>
<td>2715±68^2 a</td>
<td>2860±16^cd</td>
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<tr>
<td>Protein (% of total energy)</td>
<td>47.1±8.3^abc</td>
<td>47.6±2.2^de</td>
</tr>
<tr>
<td>Arginine (g)</td>
<td>3.8±2.3^a</td>
<td>3.1±0.4^c</td>
</tr>
<tr>
<td>Glutamine (g)</td>
<td>11.7±6.4^a</td>
<td>14.0±2.2^bc</td>
</tr>
<tr>
<td>Omega 3 (g)</td>
<td>1.0±0.6^ab</td>
<td>1.8±0.4^cde</td>
</tr>
<tr>
<td>Vit A (IU)</td>
<td>6096±6941</td>
<td>1829±265^a</td>
</tr>
<tr>
<td>Vit D (IU)</td>
<td>143.0±99.1^abc</td>
<td>11.3±8.0^a</td>
</tr>
<tr>
<td>Vit C (mg)</td>
<td>134.2±113.2^a</td>
<td>67.7±39.4^a</td>
</tr>
<tr>
<td>Zinc (mg)</td>
<td>10.0±4.8^abc</td>
<td>6.5±1.2^a</td>
</tr>
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Independent samples t-test was used to determine differences in nutrient intake between SR and SR+ during each study period (i.e., indicated in the column headings); *indicates significant difference from SR, p<0.05; **indicates significant difference from SR, p<0.0001. ANOVA was used to determine within group differences between each study period (e.g., live-in and post-study). Similar superscript letters indicate significant within group differences for each nutrient (p<0.05).
Figure 1. Timeline of activities during adequate sleep (A) and sleep restriction (B) phases. 
1Dietary intervention during sleep restriction consisted of 0.8 g protein/kg body weight plus placebo beverage (SR) or 0.8 g protein/kg body weight plus multi-nutrient beverage (SR+).
Figure 2. Photographs of the suction blister template, the subsequent blisters and the wound fluid collection template.
**Figure 3:** Circulating concentrations of serum C-Reactive Protein (CRP) Growth Hormone (GH), Cortisol, and Cytokines.

SR = blisters were induced after 48-h of sleep restriction in participants who underwent 72-h of total sleep restriction (monitored in laboratory with ~2-h sleep per night) without additional dietary protein or multi-nutrient beverage; SR+ = blisters were induced after 48-h of sleep restriction in participants who underwent 72-h of total sleep restriction (monitored in laboratory with ~2-h sleep per night) with additional dietary protein or multi-nutrient beverage. Linear mixed models with first order autoregressive covariance type was used to determine main effects of time and condition, and their interactive effects. When significant main effects or interactions were observed, all possible t-tests were conducted and the Bonferroni correction was used to control the familywise error rate. Values are means ± SD. $^a$significant within group difference from Day 1 (p<0.05); $^b$significant within...
group difference from day 4 (p<0.05); 1significantly different from SR+ (p<0.05), *indicate significant difference between groups at specified time-points (p <0.0001).
Figure 4: Cytokine response of wound exudate in participants who underwent 72-h sleep restriction with and without nutrition intervention.

SR = blisters were induced after 48-h of sleep restriction in participants who underwent 72-h of total sleep restriction (monitored in laboratory with ~2-h sleep per night) without additional dietary protein or multi-nutrient beverage; SR+ = blisters were induced after 48-h of sleep restriction in participants who underwent 72-h of total sleep restriction (monitored in laboratory with ~2-h sleep per night) with additional dietary protein or multi-nutrient beverage.  a = significantly different form 4 Hr time point (p<0.05), b = significantly different from 7 Hr time point (p<0.05), c = significantly different from 24 Hr time point (p<0.05), 1 = indicates significant between group difference at specified time-point (p<0.05). Linear mixed models with first order autoregressive covariance type was used to determine main effects of time and condition, and their interactive effects. When significant main effects or interactions were observed, all possible t-tests were conducted and the Bonferroni correction was used to control the familywise error rate. Independent samples t-test was used to determine differences between groups in terms of total cytokine concentrations (AUCs). Values are means ± SD.*indicates significant differences between SR and SR+ in terms of AUCs.