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14. ABSTRACT

This report results from a contract tasking University of Oxford as follows: The contractor will investigate the effects of limited sleep loss on the depression of immune function, as well as the use of glutamine supplementation to restore or enhance immune function.

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SLEEP DEPRIVATION IN HUMANS AND TRANSIENT IMMUNODEPRESSION

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SUMMARY

Objective: To determine (a) if the loss of one night's sleep (8 hours) leads to transient immunodepression, (b) if so, then if this immunodepression is moderated by the following night's normal sleep, (c) if not, then if this immunodepression may be moderated by the second night's normal sleep, and (d) if not, then if the provision of glutamine (ca. 0.1g/kg body wt) may restore or enhance immunocompetence in some cells of the immune system.

Aircrews and other personnel often lose sleep due to night operations, long-haul flights and shift work. Other individuals can be similarly affected for other reasons, e.g. hospital patients. It is important to know whether, in these circumstances, immunodepression is a problem.

INTRODUCTION

Civilian and military aircrews and ground personnel often lose sleep due to night operations, long-haul flights, and shift work. Other sections of the community can be similarly affected for other reasons, e.g. patients in hospital. It is important to know whether, in these circumstances, immunodepression is a problem that might lead to additional complications to the psychological stress of adjusting to a different biological rhythm.

Sleep and Immunocompetence

In a civil aviation study, air crews flying long distance trans-meridian routes reported more health problems than short-distance personnel; these problems affected more than 30% of personnel (both pilots and cabin staff) and included colds, fatigue and sleep disturbances (Haugli et al., 1994).

Increasing evidence suggests that sleep is important for proper function of the immune system (Rechtschaffen et al., 1983). Sleep deprivation can lead to immunodepression and impaired health (Benca & Quintas, 1997; Naitoh et al., 1990). Humans susceptible to the effects of sleep deprivation in different situations include: shiftworkers (Miller, 1992), intensive care patients (Schwab, 1994), infants (Kahn et al., 1994), ship crews and submariners (Miller et al., 1999; Miller et al., 2002).

Sleep disturbance is a serious problem in hospital patients. Yarrington & Mehta (1998) referred to the importance of deep sleep in promoting recovery after bone marrow transplant. They commented on the therapeutic effects of melatonin, which enhances immune response and protects against viral infections. Post-operatively, slow-wave sleep was found to be substantially decreased in patients for several days after major surgery (Orr & Stahl, 1977).

Studies of sleep-deprived animals have shown a progressive, chronic negative energy balance and gradual deterioration of health, culminating in fatal bloodstream infection without an infectious focus. In *in vitro* studies on sleep-deprived rats, Everson & Toth (2000) observed early infection of the mesenteric lymph nodes due, apparently, to bacterial translocation. The authors suggest that bacterial translocation leading to septicemia and death is the mechanism by which sleep deprivation may affect health adversely (but see Rechtschaffen & Bergmann, 2001).

A few studies have investigated the effects of sleep deprivation on some aspects of immune cell function. For example, Benca et al. (1989) looked at lymphocyte proliferative ability. However, the data were contradictory. In another study, the results of administering antibiotics to rats in the first four days of sleep deprivation were inconclusive (Bergmann et al., 1996). With regard to sleep quality studies, it has been suggested that subjective sleep quality is linked with immunodepression to an extent that is not accounted for by depression *per se* (Savard et al., 1999). They found that the amount of sleep obtained was significantly associated with the circulating numbers of CD4 and CD8 cells. White et al. (1995) observed that sleep structure distortion was a consistently replicable physiological sign of infection in HIV patients. They

found CD4 cell numbers associated with a distortion in nocturnal sleep and suggested this might be linked with immune function. In 1989, Brown et al. observed in rats that an 8-hr period of sleep deprivation led to the suppression of secondary antibody response. This suppression was reversed with administration of IL-1b (a pro-inflammatory cytokine which induces sleep) or muramyl dipeptide prior to sleep deprivation. Vgontzas et al. (2001) observed increased ACTH and cortisol secretion in the evening and first half of the night in male and female insomniacs. The highest incidence of sleep disturbances correlated with the highest amount of cortisol secreted during a 4-night sleep study.

However, there are comparatively few detailed studies on specific immune function parameters in humans that provide convincing evidence of a link between sleep deprivation/disorders and immunodepression (see Naitoh et al., 1990). The present investigation planned to address those areas where there are deficiencies to provide further evidence for or against such a link, starting with acute sleep deprivation.

Glutamine and Immune System Cells

An important fuel for some key cells of the immune system is the amino acid, glutamine. Glutamine is the most abundant amino acid in the body. It was originally classified as a non-essential amino acid, being synthesized predominantly by skeletal muscle. However, evidence is growing that glutamine is a conditionally essential amino acid (Lacey & Wilmore, 1990). As well as providing energy, glutamine is a nitrogen donor for purine and pyrimidine nucleotide synthesis. This is essential for the synthesis of new DNA in, e.g. lymphocyte proliferation, or DNA repair and mRNA synthesis in macrophages (Ardawi & Newsholme, 1983; 1985). The concentration of plasma glutamine (p[Gln]) is decreased during stress, e.g. after major surgery, burns, or prolonged, intensive exercise (Askenazi et al., 1980; Parry-Billings et al., 1990, 1992; Castell et al., 1996). In vitro studies have shown that, despite the presence of all other nutrients including glucose, only altering the glutamine concentration in the medium led to a reduction in lymphocyte proliferative ability. This was coupled with a decrease in response time of the cells (Parry-Billings et al., 1990).

Immune Function, Strenuous Exercise and Glutamine

The provision of glutamine, or of the branched chain amino acids (BCAA) as glutamine precursors, may help to combat transient immunodepression observed following sleep deprivation. Prolonged, exhaustive acute exercise and training can lead to a high incidence of upper respiratory tract illness (URTI) (Peters & Bateman, 1983) and to immunodepression (see Pedersen & Hoffman-Goetz, 2000). These exercise-induced changes can be exacerbated by factors such as exposure to hostile environments, thermal stress and sleep disturbances. Altered sleeping patterns are also a well-known problem in overtrained athletes, who suffer a high incidence of minor illnesses /infections (Budgett, 1994). Parry-Billings et al. (1992) observed a lower p[Gln] in overtrained athletes at rest compared with fit, elite athletes. Transient immunodepression occurs in endurance athletes due to the stress of undertaking prolonged, exhaustive exercise (see Nieman & Pedersen, 1999). This leads to a high incidence of illness,

particularly upper respiratory tract infections (URTI). An important factor in this situation could be lower levels of glutamine in the blood, leading to lack of availability for some immune cells at a time of stress (Newsholme & Castell, 2000).

A marked decrease in self-reported illness (34%) was reported in more than 70 marathon runners receiving glutamine compared with a similar number receiving placebo (Castell et al., 1996). These individuals had a 20% decrease in p[Gln] within one hour of finishing the race. Similar observations have been made in triathletes receiving branched chain amino acid (BCAA) supplementation (Bassitt et al. 2000). They observed a 40% decrease in the incidence of infections as well as increased lymphocyte proliferative ability and enhanced cytokine production. Bassitt et al. attributed these findings to the maintenance of (p[Gln]) via BCAA precursors. In a recent study, a decrease was observed in the incidence of infections in marathon runners who took BCAA for four weeks prior to a race (Hiscock et al., 2001).

Central Fatigue

In addition to BCAA acting as precursors for p[Gln], they have a role in central fatigue, which emanates from the brain, as opposed to peripheral fatigue, which emanates from muscle.

Tryptophan is the precursor for the neurotransmitter 5-hydroxytryptamine (5-HT), which is involved in fatigue and sleep. It is present in bound and free form in the blood, where the concentration is controlled by albumin binding to tryptophan, which competes for binding with free fatty acids (non-esterified fatty acids, NEFA). Increased mobilization of NEFA in a stressful situation due to a surge in catecholamines results in more free tryptophan in the circulation. An increase in plasma free tryptophan leads to an increased rate of entry of tryptophan into the brain. This will result in an increased rate of synthesis of 5-HT, leading to a higher concentration of brain 5-HT, which may cause central fatigue. Central fatigue is implicated in clinical conditions such as chronic fatigue syndrome and post-operative fatigue after major surgery (McGuire et al., 2001). Increased plasma free tryptophan leads to an increase in the plasma concentration ratio of free tryptophan to the branched chain amino acids (BCAA) which compete with tryptophan for entry into the brain across the blood-brain barrier. During prolonged exercise BCAA are taken up by muscle from the circulation. Thus, the provision of BCAA will increase the plasma concentration of BCAA (p[BCAA]). This may help to combat the surge in free tryptophan, ultimately decreasing the concentration of brain 5-HT.

Psychological aspects of fatigue

There is a considerable overlap of symptoms between chronic fatigue syndrome (CFS) and the overtraining syndrome (recently redefined as Unexplained Underperformance Syndrome – UUPS, Budgett et al., 2000). In addition to fatigue, these symptoms include lack of motivation, labile mood and a high incidence of minor infections. This latter suggests possible immunodepression in these individuals. In a recent study on rowers receiving BCAA daily for one month during training, Hiscock and Castell (2001) found a small increase in lymphocyte proliferation. They also observed a significant effect in the alleviation of depression in those rowers taking BCAA compared with the placebo group.

In elite cross-country runners, who were also U.S. Air Force Academy cadets, correlations have been observed between cognitive hardiness, immune cell numbers and function (Drummond et al., 2001). The present study investigated whether a link between fatigue, immunodepression and mood might be exacerbated by sleep deprivation for one night, and whether this could be ameliorated by the restoration of normal sleep or intervention.

METHODS

The aim of Study 1 (a pilot study) was to determine (a) if the loss of one night's sleep (8 hours) leads to transient immunodepression; (b) if so, then whether this immunodepression could be moderated by the following night's normal sleep, and/or a second night's normal sleep. In (Study 2), glutamine (ca. 0.1g/kg body wt) was given daily for 4 days in order to ascertain whether it moderated those aspects of immunodepression observed in Study 1.

Experimental Design

The design involved two components, with the same design applied to each of two experiments. First, we apportioned variance within biochemical, haematological, performance and subjective measures across four mornings of repeated testing, and then compared the four sets of results to each other. Second, we acquired several repeated performance and subjective measures hourly across a single night of sleep deprivation (preceding the second morning) and described measure distributions for each hour's measurement session.

Study 1 was designed to accomplish the objectives above on 12 healthy volunteers.

Study 2 was designed to repeat Study 1 on 16 subjects but with the additional component of feeding glutamine vs. placebo. These subjects took daily oral doses of placebo (n = 8) or glutamine (n = 8), 1 x 5 g per day (double blind). Glutamine and an identically packaged placebo (maltodextrin) were purchased from Oxford Nutrition, Witney, UK. The glutamine and placebo both contained aspartame for sweetening and less than 2% phenylalanine.

Statistics

Study 1: A sample size of twelve was selected to provide a test power of 0.91 for a two-tailed test for an effect size of 1 standard deviation unit, with a confidence level of 0.95 and a test-retest reliability of 0.50 (Cohen, 1988).

Study 2: The sample size of eight subjects per week for two weeks provided a test power of 0.46 for a two-tailed test for an effect size of 1 standard deviation unit with a confidence level of 0.95 and independent groups (*ibid.*).

Subjects

Healthy male subjects (Study 1, n=12; Study 2, n=16) were recruited from the local community. Applicants were excluded if they had clinical sleep problems, infectious conditions, immune compromises, and/or endocrine problems. (Subject details are set out in Table 1)

Table 1. Subject characteristics

Study 1	*Study 2*	
Parameter	*Mean (Range)*	*Mean (Range)*
Age (yr)	25 (20-33)	28 (19-42)
Height (m)	1.80 (1.7-1.9)	1.77 (1.6-1.8)
Weight (kg)	91 (70-111)	91 (70-111)
Reported alcohol (drinks/wk)	2.0 (0-6)	2.7 (0-10)
Nicotine	1 user	5 users
Sleep hr/weekday	6.4 (5-8)	6.8 (5-9)
Sleep hr/weekend	7.2 (5-10)	7.8 (5-11)
Shiftworkers	2	1

Questionnaires

Sleep Behavior Questionnaire. A Sleep Behavior Questionnaire dealing with adequacy and quality (designed by JC Miller and PA Hickey, 2000) was used. The primary design resources were questionnaires from The Scripps Research Institute Sleep Research Group, see Wylie *et al.* (1996), and from Queensland University of Technology (Hubinger, 1998).

Morningness-Eveningness. The Morningness-Eveningness Questionnaire (Horne and Ostberg, 1976) was designed to help reveal tendencies toward morning (lark) or evening (owl) circadian rhythm patterns.

Sleep Hygiene and Practices Survey (SHAPS). The SHAPS survey concerned subjects' knowledge of the effects (1 to 7 scale) of selected daytime behaviors upon sleep and of the presence of caffeine in various over-the-counter medications, food and drink (Lacks and Robert, 1986).

Beck Depression Inventory. The Beck Depression Inventory is a 21-item self-report rating inventory measuring characteristic attitudes and symptoms of depression (Beck *et al.*, 1961 revised in 1971). The trait anxiety score was also administered as a potential covariate for analyses of sleep quantity and quality data.

Trait Anxiety Inventory. The State-Trait Anxiety Inventory (STAI; Spielberger, 1983) is designed to differentiate between the temporary condition of "state anxiety" (see below) and the more general and long-standing quality of "trait anxiety" in young adults. The STAI is used widely in sleep disorder centers because it is easily administered and interpreted and it targets a psychopathology that is commonly associated with insomnia (Spielman *et al.*, 2000).

Cognitive Hardiness Scale. Cognitive hardiness (CH) is a sense of control, commitment to the projects and people in one's life (Kobasa, 1982), and a tendency to appraise events as challenges (versus threats). CH appears to moderate the relation between stress and both illness and depression, and has predicted cortisol reactivity. The CH score was administered as a potential covariate for analyses of performance data.

Epworth Sleepiness Scale. The Epworth Sleepiness Scale (ESS; Johns, 1991, 1992) was also used as a potential covariate for analyses of performance data.

Incidence of Illness Questionnaire. An Incidence of Illness self-reporting questionnaire was given to the subjects on the first morning of the study. Symptoms queried included cold, cough, sore throat, flu, diarrhea, fever, headache. Subjects were asked to note if they experienced any of the listed symptoms during, and for three days after, the four-day study.

Performance Tests

Simple Cognitive Performance Battery. A cognitive performance test battery was implemented on desktop personal computers in the Windows® operating system using the Navy's Automated Neuropsychological Assessment Metrics (ANAM) library. It consisted of a library of tests and batteries designed for a broad spectrum of clinical and research applications. This library of computerized tests was constructed to meet the need for measurement of cognitive processing efficiency in a variety of psychological assessment contexts that include neuropsychology, fitness for duty, neurotoxicology, pharmacology, and human factors research (Reeves *et al.*, 2001).

All stimuli were presented on the PC screen, and all performance task responses were made with the PC mouse buttons with the preferred hand. The battery included the following tests.

- Simple response time task: simply required a rapid mouse-button press in response to the display of the ∗ symbol. There were 20 trials, with an interstimulus interval that varied from 650 to 1100 msec. Timeout (no response) occurred at 1000 msec.
- Mental arithmetic task: required a left or right click corresponding to a < 5 or > 5 solution of an addition-subtraction problem consisting of three single digits. The probe duration was set to 4500ms, with a timeout value of 5000ms. As soon as the subject responded another probe was presented. The task ran for three minutes.
- Logical reasoning task: required a left or right click corresponding to a true-false choice about a positive or negative statement concerning the order of two symbols. The probe duration was set to 4500ms, with a timeout at 5000ms. As soon as the subject responded another probe was presented.

Task training on the ANAM was conducted during the week immediately preceding the Experiment. The subjects completed the ANAM battery six times during training, with two additional sessions on the logical reasoning task. The ANAM test order was Simple Response Time (SRT), Mental Arithmetic and Logical Reasoning.

Measures acquired from these tasks included percent accuracy, mean response time for correct responses (MNRTC), numbers of omissions, standard deviation of response time for correct responses (SDRTC), and throughput (number correct divided by mean response time for all responses, in units of number correct per minute)

Vigilance Performance. Vigilance performance was assessed using the Psychomotor Vigilance Task (PVT; Dinges, 1992; Dinges *et al.*, 1997) (Vigilance Task Monitor, Model PVT-192, CWE, Inc., Ardmore PA, available from Ambulatory Monitoring, Inc., Ardsley NY). Task training on the PVT was conducted during the week immediately preceding the Experiment. The subjects completed the 10-min PVT at least once during training

Subjective Measures

Stanford Sleepiness Scale. The Stanford Sleepiness Scale (SSS; Hoddes *et al.*, 1973) correlates with standard measures of performance and usually reflects the effects of sleep loss. Horne (1991) suggested parallelism between the SSS and the alertness-sleepiness descriptors used for the "vigor" factor of the Profile of Mood States (POMS). The POMS vigor scale has also demonstrated sensitivity and reliability with respect to quantifying perceptions of sleepiness.

State Anxiety Inventory. The State Anxiety Inventory (STAI; Spielberger, 1983) is designed to evaluate feelings of apprehension, tension, nervousness, and worry, which increase in response to physical danger and psychological stress. This test was used repetitively during the 4 days of testing.

The Profile of Mood States (POMS). The POMS questionnaire (Education and Industrial Testing Service, San Diego,1971) was used to measure dimensions of affect or mood. It consisted of 65 adjectives describing feeling and mood to which the subjects responded according to a five-point scale ranging from "Not at all" to "Extremely." These are summed under the headings "Tension, Depression, Anger, Vigor, Fatigue and Confusion. Standard instructions were to indicate mood reactions for the "past week including today."

Physiological Measures

Body Temperature. Oral temperature was taken hourly during the night of sleep deprivation to estimate circadian rhythm in body metabolism. The subjects were instructed to refrain from eating or drinking for 15 min prior to scheduled temperature measurements, and proctors monitored this behavior.

Activity. The ActiWatch (WAM Inc.,) wristwatch has a small piezoelectric accelerometer which systematically records the individual's movement over time, both while awake and asleep. Subjects were instructed to wear the WAM on the wrist of the non-preferred hand (removing their own watch). They were asked to wear it constantly but to avoid water immersion and any activity where it might impede performance. Actigraphy data were used to estimate the sleep patterns of the subjects for 24 hrs after the night's sleep deprivation. The data were reduced using the Cole-Kripke sleep scoring algorithm (Cole *et al.*, 1992) to categorize each recorded epoch into sleep and awake periods.

Subjects were asked to use the Activity Log (devised and used in this laboratory for a number of years) to indicate for each 30min of the three successive days before the Experiment, whether they were sleeping or trying to sleep.

Blood samples. Blood samples were taken resting, fasting, with venestasis, into four Vacutainers (Becton Dickinson): 2 x Lithium Heparin; 1 x EDTA; 1 x No anti-coagulant (total of 23ml). The serum samples (no anti-coagulant) were kept at room temperature; one set of Li Hep tubes was put on ice prior to centrifugation at 10^{o}C. Both sets were centrifuged for 12 min at 600xg, and the serum and plasma were aliquotted into 3 Eppendorf tubes respectively and stored at -80^{o}C. EDTA samples had full blood counts performed and the ratio of CD4 (helper) to CD8 (suppressor/cytotoxic T-cells) was determined on a Coulter Counter (EPICS).

Blood biochemistry. Serum was assayed for cortisol, via Immunoradiometric assay (IRMA); and for cytokines IL-6, IL-1β and IL-8 using a flow cytometry bead assay which has the advantage of using very small quantities to measure a large number of cytokines simultaneously (Dr Carl Stewart's laboratory, Rockefeller Institute, New York). Plasma was assayed for anti-oxidant capacity using a chemiluminescent technique (Pholasin^{R}, Knight Scientific Ltd, Plymouth, U.K.); for glutamine enzymatically (Windmueller & Spaeth, 1974).

Procedures

Day 1: Subjects arrived at the laboratory at 06:30 after a 10-hr fast and gave a 20 ml blood sample. They completed one set of ANAM cognitive tasks, the SSS and the POMS, ate breakfast in the lab, spent Day 1 at work or home, and returned to the lab by 18:00, without their vehicle. They ate dinner and then remained awake all night, fasting from 20:30-06:30. Oral temperatures and psychomotor vigilance tasks (PVT) were undertaken hourly all night.

Day 2: After a fasting blood sample at 06:30, they completed one set of ANAM cognitive tasks, the SSS and the POMS and ate breakfast before being sent to home or work either by taxi or by their own designated driver. The night of Day 2 was scheduled as normal sleep.

Days 3 and 4: The following two mornings, after an 06:30 fasting blood sample at the laboratory, subjects completed one set of ANAM cognitive tasks, the SSS and the POMS, ate breakfast and left the laboratory. The subjects' recovery sleep patterns were monitored by actigraphy across the 48 h starting at 06:30 on Day 2.

In some of the following presentations of data in the Results section, Greenhouse-Geiser (GG) adjustment was made to the degrees of freedom to deal with the presence of repeated measures in the design. The (2.522, 22.695) numbers are the GG-adjusted degrees for freedom for the calculation of significance of the F statistic. MSe is the mean squared error, the quotient of the sum of squares and n for the error term.

RESULTS

Participant Characteristics

Study 1. By all measures, the participant sample in Study 1 appeared to be a collection of normal males. None appeared to have obvious clinical problems with depression, anxiety, insomnia or excessive daytime sleepiness. Their reported sleep time was approximately normal. In a National Sleep Foundation poll taken in the year 2000, those surveyed reported sleeping about seven hours a night on the average. About one-third surveyed tended to sleep eight or more hours, and one-third tended to sleep 6.5 hours or fewer. One participant in Experiment 1 was classified as an extreme "lark."

The highest education level attained by participants was as follows: two participants had received high school diplomas, one had received a GED, one had received an Associates degree, three had received Bachelors degrees, and three had received Masters degrees.

Reported Sleep Length. The participants reported weekday nocturnal sleep lengths of 5 to 8 hours (mean 6.4 +/- 0.84 h; median 7 h) and weekend sleep lengths of 5 to 10 h (7.15 +/- 1.72 h; median 7 h). These numbers reflect slightly abbreviated sleep lengths, from the recommended 8 h per night (per the National Sleep Foundation), and some further abbreviations along with some recovery sleep on the weekends. Generally, they probably experienced acute and, perhaps, cumulative fatigue from abbreviated sleep periods.

Reported Sleep Latency. The participants reported usual sleep latencies of 2 to 30 min (xx +/- 10.1 min). These numbers suggested that the group did not usually suffer from excessive sleepiness: they generally did not fall asleep very quickly nor unusually easily. Very sleepy individuals tend to fall asleep in about 5 min, according to the general results of the Multiple Sleep Latency Test used in sleep clinics. Four of the participants reported latencies shorter than 7.5 min, and two reported latencies greater than 20 min.

Reported Sleep Inertia. The participants reported usual sleep inertia lengths of 1 to 45 min (xx +/- 13.5 min). These numbers suggested that the group did not usually suffer from excessive inertia: they passed through sleep inertia at an expected rate and did not report great difficulty doing so. Three participants reported sleep inertia less than 5 min, and seven reported inertias lasting longer than 10 min.

Epworth Sleepiness Scale (ESS). The participants reported ESS ratings ranging from x to x on the 0-to-24 sleepiness scale (mean 7.9 +/- 1.6 ; median 7.5). A higher score represents greater sleepiness. None reported sleepiness above 15, providing no cause for concern with respect to acceptable individual job performance.

Morningness-Eveningness Questionnaire (MEQ). Scores on the MEQ were used to categorize individuals as follows (Horne and Östberg, 1976):

- Definitely morning, 70-86
- Moderately morning, 59-69

- Neither, 42-58
- Moderately evening, 31-41
- Definitely evening, 16-30

The participants, individually and as a group, exhibited central tendency with a mean score of 54.1 +/- 11.5 and a median score of 52. The scores ranged from 43 to 70, distributed as:

- Definitely morning, 1 participant
- Moderately morning, 1 participant
- Neither, 5 participants

Sleep Hygiene and Practices (SHAPS). The participants indicated moderately good sleep hygiene knowledge, good caffeine knowledge, and good sleep practices:

- Sleep Hygiene Knowledge ratings ranged from 15 to 29 on the 13-to-39 scale (mean 21.6 x +/- 4.8; median 20).
- Caffeine Knowledge ratings ranged from 38.8 to 94.4 on the 0-to-100 scale (mean 77.5 +/- 16.8; median 79.9).
- Sleep Hygiene Practice ratings ranged from 5 to 30 on the 0-to-133 scale (mean 18.7 +/- 8.7; median 18.5).

(Higher scores indicated more knowledge or less healthy sleep hygiene practices.)

Beck Depression Inventory (BDI). *[Data still to come]* The participants reported BDI ratings ranging from x to x on the 21-point depression scale (mean x +/- x; median x). A higher score represented greater depression. xx fell in the 4 to 7 range, defined as normal or as mildly depressed. xx participants scored in the 8 to 15 range, defined as approximately moderately depressed.

Trait Anxiety Inventory (TAI). The participants reported TAI ratings ranging from 20 to 43 on the 20-to-80-point anxiety scale (mean 32.4 +/- 7.4; median 34). A higher score represents greater anxiety. The trait anxiety norm for working men, aged 19 to 39 yr, is 35.55 +/- 9.76. The group data replicated this norm.

Cognitive Hardiness Scale (CH). The participants reported CH ratings ranging from 103 to 143 on the 30-to-150-point hardiness scale (mean 114.8 +/- 11.6; median 113). A higher score represents greater cognitive hardiness. The group mean and median fell near the population mean of about 106 and fell near the military mean of about 114 to 118.

***Study 2*.** The highest education level attained by participants was as follows: one participant had completed 11th grade, eight participants had received high school diplomas, three had received an Associates degree, three had received Bachelors degrees, and one had received a Masters degrees.

Reported Sleep Length. The participants reported ideal sleep times of 6 to 10 h (+/- 1.1), but reported that their usual, 24-h sleep totals ranged from 5 to 9 h (+/- 1.1, all in a single period). Thus, it appeared that they usually acquired their ideal amount of sleep, but

occasionally experienced acute fatigue from shortened sleep periods. About 1/8 of the participants reported taking occasional naps of 30 minutes to 2-hrs.

Reported Sleep Latency. The participants reported usual sleep latencies of 5 to 60 min (+/- 15.5 min). These numbers suggested that the group did not usually suffer from excessive sleepiness: they did not fall asleep very quickly nor unusually easily. Very sleepy individuals tend to fall asleep in about 5 min, according to the general results of the Multiple Sleep Latency Test used in sleep clinics. Two of the participants reported latencies shorter than 7.5 min, and nine reported latencies greater than 20 min.

Reported Sleep Inertia. The participants reported usual sleep inertia lengths of 3 to 90 min (+/- min). These numbers suggested that the group suffered slightly from excessive inertia: they passed through sleep inertia at a slightly-slower-than expected rate. One participant reported a sleep inertia less than 5 min, and thirteen reported inertias lasting longer than10 min.

Epworth Sleepiness Scale (ESS). Participant scores ranged from 4 to 16 on the Epworth Sleepiness Scale (mean 8.26 +/- 3.10; median 8.00). Ratings above 15 out of a possible 24 are cause for concern with respect to acceptable job performance. One participant scored 16.

Morningness-Eveningness Questionnaire (MEQ). The participants, individually and as a group, exhibited central tendency with a mean score of 57.31 +/- 8.1 and a median score of 56.5. The scores ranged from 43 - 68, distributed as:

- Neither, 10 participants
- Moderately morning, 5 participant
- Definitely morning, 1 participant

Sleep Hygiene and Practices (SHAPS). The participants indicated moderately good sleep hygiene and caffeine knowledge, and good sleep practices.

- Sleep Hygiene Knowledge ratings ranging from 13 - 30 on the 13-to-39 scale (mean 21 +/- 4.1; median 21).
- Caffeine Knowledge ratings ranging from 58.82 to 100.00 on the 0-to-100 scale (mean 73.76 +/- 12.97; median 71.42).
- Sleep Hygiene Practice ratings ranging from 3 to 18 on the 0-to-133 scale (mean 12.13 +/- 4.3; median 13.00).

Beck Depression Inventory (BDI). The participants reported BDI ratings ranging 0 - 13 on the 21-point depression scale (mean 3.13 +/- 4.4; median 1). A higher score represents greater depression. Eleven fell into the 0-3 range. Two fell into the 4 to 7 range, defined as normal or as mildly depressed. Three participants scored in the 8 to 15 range, defined as approximately moderately depressed.

Cognitive Hardiness Questionnaire. The participants reported CH ratings ranging from 94-137 on the 30-to-150-point hardiness scale (mean 116.8 +/- x; median 118). A higher score represents greater cognitive hardiness. The group mean and median fell near the population mean of about 106 and fell within the military mean of about 114 to 118.

Performance Tests

Study 2

SRT, N=14: **Median reaction time all**, main effect of day, F(2.455, 24.554)=3.711, MSe=171.394, GG p=0.032. **Median reaction time correct**: main effect of day, F(2.453, 24.531)=3.722, MSe=171.499, GG p=0.031.

LRS, N=13: No GG significant interactive or main effects.

Study 1

Daily Tests. There were no statistically significant effects across days for simple response time, mathematical processing or logical reasoning.

PVT Mean Reciprocal Response Time (response speed) revealed the expected main effect of trial during the night of sleep deprivation (p=0.000; Figure 1): MRRT decreased over time.

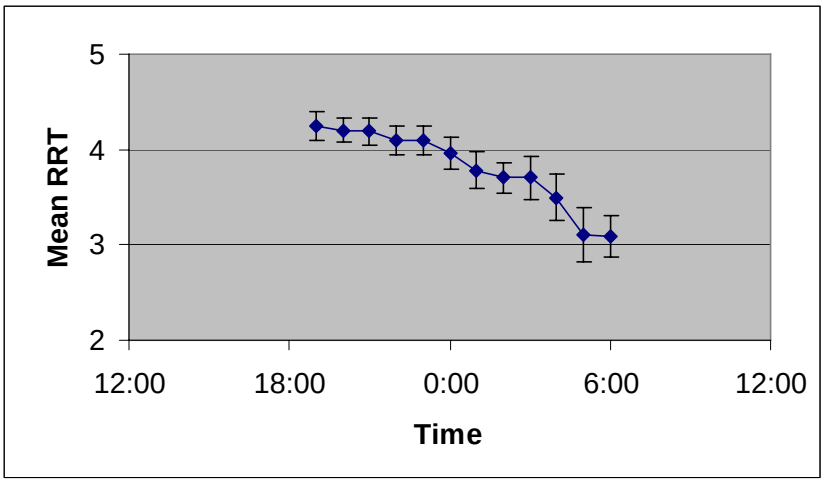

Figure 1. Overnight pattern of PVT Mean Reciprocal Response Time (1/msec +/- std err).

PVT Mean Fastest 10% Reciprocal Response Time (FRRT) revealed a similar main effect of trial (GG F(2.673, 24.055)=4.071, MSe=0.436, p=0.021; Figure 2): there was a decline during the night of sleep deprivation.

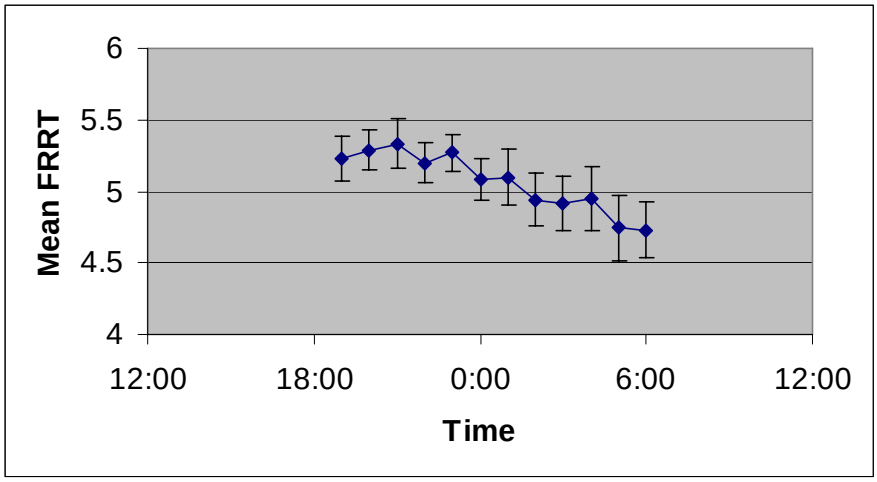

Figure 2. Overnight pattern of PVT Fastest Reciprocal Response Times (1/msec +/- std err).

PVT Mean Slowest 10% Reciprocal Response Time (SRRT) declined during the night in a similar fashion (GG F(3.899, 35.094)=13.342, MSe=0.661, p=0.000; Fig. 3).

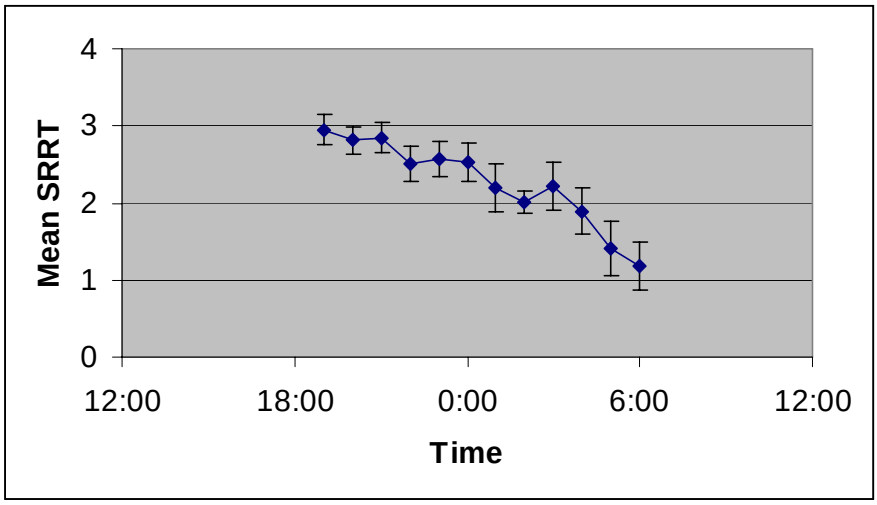

Figure 3. Overnight pattern of PVT Slowest Reciprocal Response Times (1/msec +/- std err).

PVT Lapses revealed a main effect of trial (GG F(2.022, 18.197)=6.546, MSe=136.570, p=0.007; Figure 4): the number of lapses increased across the night of wakefulness.

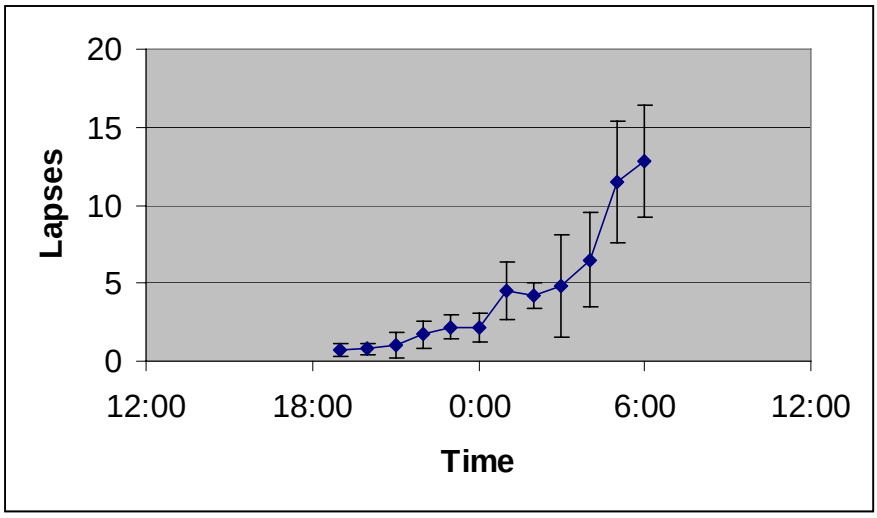

Figure 4. Overnight pattern of the PVT lapses (+/- std err).

Subjective Measures

Sleepiness

Study 1. There was a marked effect of sleep deprivation, increasing subjective sleepiness from 1.7 $\pm$0.2 on the Stanford Sleepiness Scale (SSS) to 5.3 $\pm$0.5 ($p<0.001$; Figure 5a). [parallel analyses across two studies]

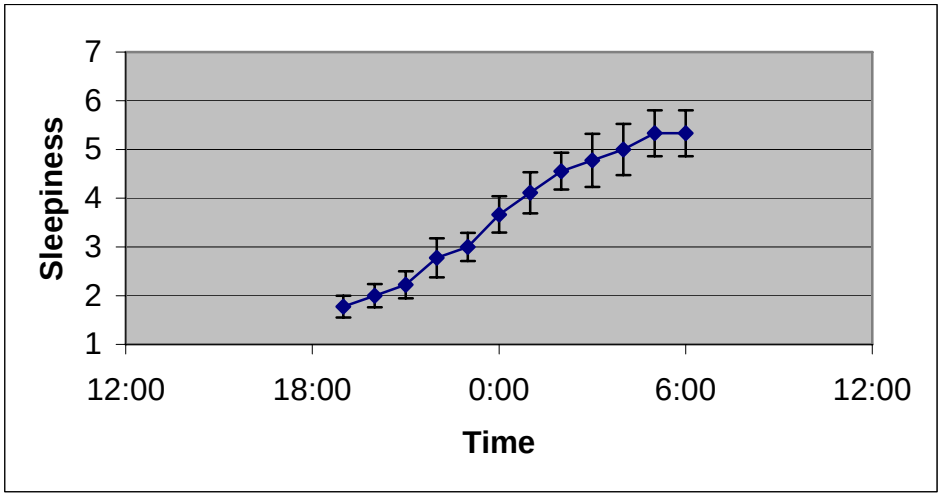

Figure 5a. Overnight sleepiness ratings in Study 1 (+/- std err).

Study 2. There was a significant main effect of trial on the sleepiness rating (GG F(4.3, 47.0)=40.433, MSe=1.226, p=0.000). Pairwise comparisons revealed the sleepiness rating for trial 1 was significantly lower than for trials 6-12, trials 2 and 3 were significantly lower than trials 7-12 and trials 4 and 5 were significantly lower than trials 8-12.

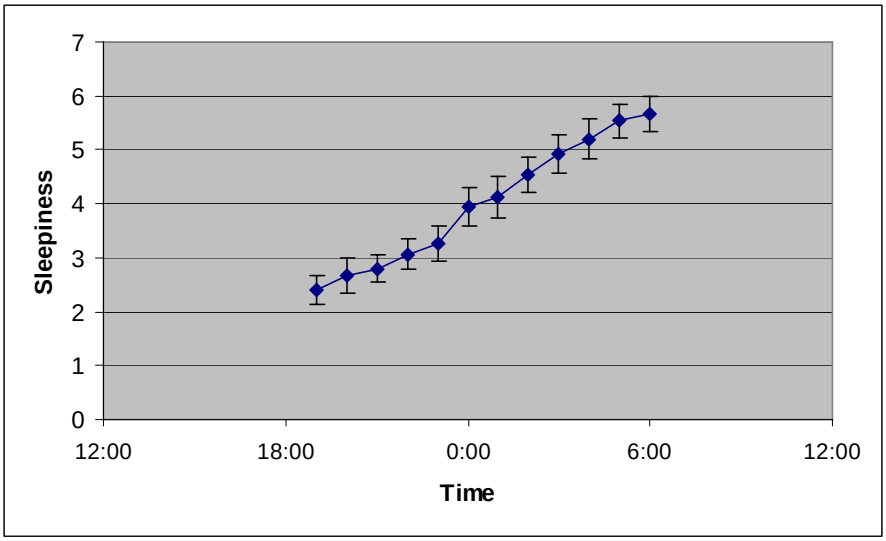

Figure 5b. Overnight sleepiness ratings in Study 2 (+/- std err).

Daily Effect in Study 2. Subjects rated their sleepiness each morning of the study. There was a significant effect of trial on the SSS (GG F(1.7, 15.6)=27.853, MSe=1.491, p=0.000; Fig. 6). The mean rating for morning two was significantly greater than mornings 1, 3, and 4, attributable to the night of sleep deprivation immediately before morning 2.

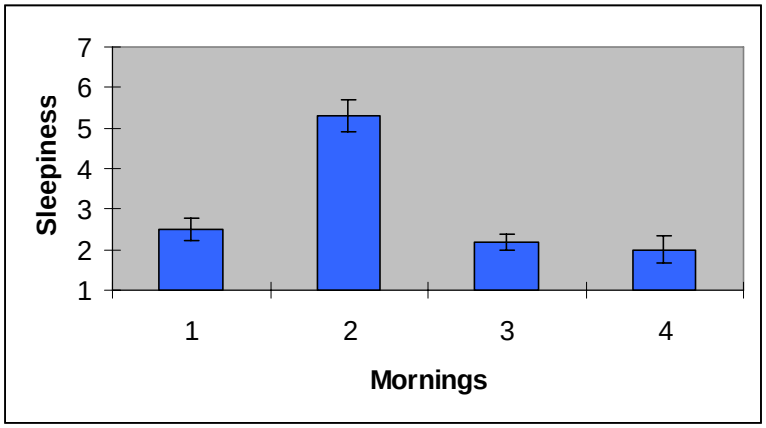

Figure 6. Effect of Days on sleepiness rating (+/- std err).

State Anxiety. A main effect of trial was observed for the State Anxiety Inventory (SAI) across the four mornings. GG(2.264,20.375)=4.964 , MSe=107.989, p=0.015. Pairwise comparisons demonstrated that trial two was significantly increased from trial one (p=0.016), and that trial two was also significantly increased over trial 4 (p=0.016)

Mood. The POMS revealed a main effect of day on three of the six subscales: Anger-Hostility, Fatigue-Inertia, and Tension-Anxiety (Figure X). There was a significant effect on Anger-Hostility (GG F(2.138, 19.241)=3.685, MSe=4.453, p=0.042): the mean ratings declined over days 3 and 4. Day also had a significant effect on Fatigue-Inertia (GG F(1.351, 12.155)=5.973,

MSe=73.351, p=0.024): Fatigue-Inertia ratings peaked during day 2 and were significantly higher than the ratings during day 4 (p=0.051). There was a significant effect on Tension-Anxiety (GG F(2.644, 23.799)=3.850, MSe=4.054, p=0.026): Tension-Anxiety ratings for day 2 were significantly higher than for day 4 (p=0.046).

Physiological Measures

Study 1
Body Temperature: There was a marked effect of one night's sleep deprivation on oral body temperature which decreased from 98.89±0.089°F to 97.29±0.218°F (p<0.000) (Fig. 7).

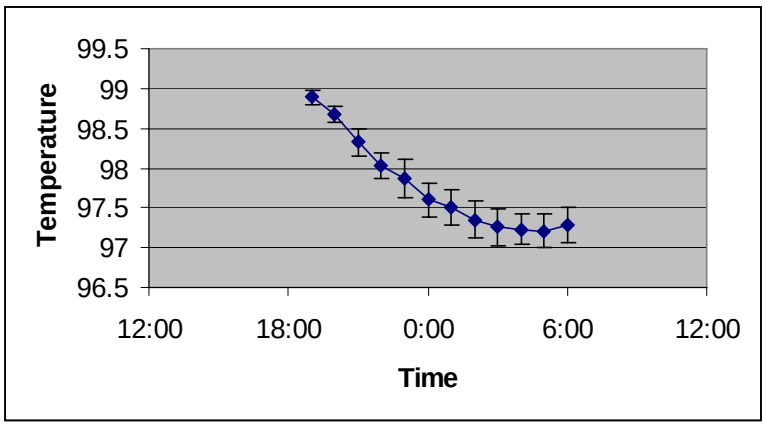

Figure 7. Overnight pattern of oral temperature (deg F +/- std err).

Activity (ActiWatch Activity Log): Based on the activity log, the ten subjects averaged 7.35 hours of sleep during the 96 hours prior to the study. The sleep quality ratings included: 8 extremely good, 24 moderately good, 2 moderately poor, and 6 no responses. Two subjects took two naps each (3 & 2 hours; 2 & 2 hours, respectively) during the 96 hours prior to the study.

Incidence of Illness
Study 1: Four subjects experienced headaches: two on the first day of the study, two on the second day, and two on the third day of the study. One subject experienced sinus pain during Days 1-3. One subject experienced left ear pain on Days1-4. No subjects had cold, cough, sore throat, flu, diarrhea, or fever during Days 1-4.
Study 2: 54% of subjects reported having no illness throughout the study. Of the remainder, 38% had a fever or a cough for 1-2 Days only. One subject was ill with fever and a runny nose two days before, during and for three days after the study. There was no difference in the incidence of illness between groups.

Haematological data
Study 1: There were no statistically significant effects of day on the percentage or number of basophil cells, the CD4/CD8 ratio, the CD56 factor, the number of eosinophil cells, haemoglobin value, haematocrit ratio, percentage of lymphocytes, average volume of individual red blood cells (MVC), number or percentage of monocyte cells, the number or percentage of neutrophils, or the white blood cell count.

Eosinophil Percentage. A main effect of trial was observed for the percentage of eosinophil cells in the blood sample (GG(2.522,22.695)=3.061, MSe=0.905, p=0.045). Pairwise comparisons indicated a significant decrease in the percentage of cells from Day 2 to Day 3 (p=0.086).

Lymphocytes: A main effect of trial was observed for the number of lymphocyte cells found in the blood samples (GG(2.325,20.928)=5.457, MSe=0.408, p=0.010). Pairwise comparisons demonstrated a significant decrease between Days 2 and 3 (p=0.071), as well as Days 2 and 4 (p=0.085). Number of lymphocytes was lower in Gln group at Day 3.

Study 2: Mean red blood cell volume was lower on Day 2 (p=0.002) for both groups
Percent neutrophils was lower in Gln at Day 2 ($p<0.05$)

Non-significant trends:
a). The percentage and number of basophils were nearly two-fold higher in the Gln group at Day 2 ($p<0.08$);
b). Number of monocytes was higher in Plac throughout the study; monocyte percentage was markedly higher in Plac vs. Gln at Day 4;
c). Number of platelets was higher at Days 3 & 4 in Gln;
d). Total white blood cell numbers were slightly higher in Plac throughout the study;
e). The CD4/CD8 cell ratio was decreased at Day 3 in both groups;
f). Percentage of CD56 positive cells were lower in Plac throughout the study.

Cytokine analysis: *(Not measured in Study 1).* In *Study 2:* serum cytokines (IL-1, IL-6, IL-8) were unaffected in most subjects. One subject, who reported fever and cold throughout the study, had very high levels of serum IL-6 and IL-8.

Serum leptin: *(Not measured in Study 1).* In *Study 2*: eight out of fourteen subjects, serum leptin decreased (by 24±4%) at Day 2. In two subjects there was no change, two were markedly increased. There was no effect of Plac or Gln. Leptin is linked with sleep disturbance, exercise fatigue, behaviour and cognition.

Serum cortisol data: *(Not measured in Study 1).* *In Study 2 :* There was a 28% increase in serum cortisol concentration at Day 2 ($p<0.001$) compared with baseline, and an 18% increase at Day 4 ($p<0.1$).

Oxidative stress data: There were no significant changes or between group differences in antioxidant capacity.

Amino acid analysis: There was a non-significant trend towards a decrease in p[Gln] at Day 2 in Study 1, which was maintained throughout the study. In Study 2 there was a close-to-significant decrease in p[Gln] (of 10%, $p<0.1$) at Day 4 in both Gln and Plac groups. A non-significant trend towards a decrease in the plasma glutamine concentration (p[Gln]) on Day 2 in the placebo group was observed. There were no significant changes in p[Gln] in relation to either time or group.

DISCUSSION

One night's sleep deprivation had a marked effect on sleepiness and cognitive function tests, particularly in terms of speed of response in both studies. All subjects also became increasingly sleepy during the night of sleep deprivation. Unfortunately it was not possible to control whether or not the subjects napped during the day after the sleep deprivation. This is an important issue which we plan to address in future studies. Changes in mood were reported, particularly in relation to Tension-Anxiety and Fatigue-Inertia, both of which increased after the night's sleep deprivation.

The data from both studies demonstrated that this acute sleep deprivation had a significant effect on circulating numbers of white blood cells. Lymphocytes, neutrophils and the percentage differential of eosinophils were decreased, as was the CD4/CD8 cell ratio. There were several very interesting, non-significant trends in cell data which certainly merit further investigation. Of particular interest was the apparent effect of glutamine feeding on basophils, the numbers and percentage of which doubled in the glutamine group on Day 2.

The effect of acute sleep deprivation on serum leptin in some individuals may be linked with the marked changes observed in cognitive function. There may be a link between leptin and caffeine intake and it is proposed to investigate this further. The increase in serum cortisol observed after the night's sleep deprivation appears to be a marker of stress due to loss of sleep. The night's sleep on Day 2 is likely to have compensated for loss of the previous night's sleep, and thus responsible for restoring Day 3 plasma cortisol to baseline levels observed on Monday a.m. The reason for the increase on Day 4 is less clear. Samples were always taken between 6 and 6.30a.m., thus there is unlikely to be an effect of diurnal variation.

In Study 1, a mean decrease of 8% was observed in p[Gln] in eight out of ten subjects at Day 2. Combining data from both Study 1 and the placebo group in Study 2, produced a non-significant trend towards a decrease in p[Gln] at Day 2, which remained lower during Days 3 and 4. In Study 2, no difference was observed between the Gln and Plac groups at any stage. Plasma glutamine is customarily elevated within 30-40min of ingestion (Castell, 2003). Samples were taken daily before the glutamine supplementation was given and an increase would not be expected at that time. It may be that a 5g oral bolus dose is insufficient to raise p[Gln] over several days, and that 10g twice daily would be more effective. Nevertheless, the provision of 5g Gln daily is likely to have a link with the reduction in neutrophil numbers observed after acute sleep deprivation. This may be linked to a decrease in cell production of the neutrophil chemoattractant, IL-8, which has been induced by glutamine feeding in several studies of athletes (see Castell, 1996; 2003) and patients (O'Riordain et al., 1997). In future studies, neutrophil function will be measured together with cytokine production, specifically IL-8.

NOTES

(1) It is worth noting the fact that using uncorrected values from one assay gave rise to a marked and significant decrease in p[Gln] at Day 2 in Study 1: however, because the assayed standards were lower than expected, a correction factor was employed, as is our custom, which subsequently produced a non-significant trend only. The low standards could also be attributable to a problem with the stock concentration, thus negating the need for a correction factor.

(2) Using a complex technique, involving nuclear magnetic resonance spectroscopy (Metabonomics) to which the PI's laboratory in Oxford has access, serum amino acid profiles will be measured. This will enable us to see any changes in all amino acids, including those known to be linked with immunodepression. Unfortunately, due to personnel changes this will not now be carried out until after the New Year. Because of the complexity of the assay and the calculations using a new pattern recognition technique, the data will not be available for several weeks after measurement.

(3) Further calculations need to be made on some of the psychological and performance tests used. The researcher who was dealing with this aspect of the project has moved following maternity leave. Dr Miller and another member of the team are working on this aspect of the project and will be completing the calculations in the New Year.

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