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TITLE: Randomized, Controlled Trial of Combination Treatment with Pyridostigmine,  
DEET, and Teremethrin

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<b>14. ABSTRACT</b> Background: Studies exposing animals to very high levels of the insect repellents DEET and permethrin, along with the nerve agent phylactic pyridostigmine bromide (PB), suggested synergistic toxicity; this led to hypotheses regarding "Gulf War Syndrome". Methods: A prospective, multi-center, double blind, randomized placebo-controlled trial exposed healthy humans to permethrin-impregnated uniforms, DEET-containing skin cream, and oral PB, in a manner consistent with current U.S. military doctrine. A block-randomized crossover design required 4 different sessions, ensuring exposure to all treatments and placebos under both stress (one-hour treadmill march wearing heavy backpack, simultaneously viewing combat scenes and solving math problems) and rest conditions. Physical outcome measures included handgrip strength and duration, step-climbing, and pull-ups; neurocognitive outcomes include measures of reaction time, attention span, and short term memory, employing NASA's validated computer-based WINS CAT. Results: 64 subjects completed the full study (4 sessions); 17 more completed 1-3 sessions each. Permethrin was undetectable in the serum of all subjects, while DEET and PB were readily detectable, without significant differences under stress compared to rest. Heart rate and systolic blood pressure increased significantly with stress compared to rest, but did not vary with treatment compared to placebo. Physical and neurocognitive outcome measures did not significantly differ by exposure combination. Conclusions: Permethrin applied to Battle Dress Uniforms is not detectable in the blood. Combined preventive treatment with PB, DEET, and permethrin appears safe when used as directed by men and women, under rest or stress conditions, without physical or neurocognitive impairment. Individuals requiring protection against both nerve agents and insects need not fear using these measures.					
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## **Introduction**

The U.S. military uses the insect repellents DEET and permethrin. If enemy nerve agent use is threatened, pyridostigmine bromide (PB) is also used, to reversibly inhibit 20-40% of neuromuscular junction acetylcholinesterase (AChE), preventing irreversible binding by nerve agents. Animal and human studies had demonstrated safe independent use of PB, DEET, and permethrin. However, some blamed the combination for a variety of ill-defined symptoms reported by some Gulf War veterans. One study reported synergistic neurotoxicity in chickens subjected to very large doses of pyridostigmine, DEET and permethrin. Another study indicated that PB crosses the blood-brain barrier in stressed—but not non-stressed—mice. Previous human studies simply asked about “CNS symptoms”, and were subject to selection and/or recall biases. An Institute of Medicine panel concluded “studies are needed to resolve uncertainties about whether PB, DEET, and permethrin have additive effects”. Sample size calculations indicated that 64 participants would allow sufficient power to determine whether significant differences in neurocognitive or physical outcomes could be identified with treatments compared to placebo controls. The target number of 64 healthy young volunteers was achieved, along with another 17 who completed between one and three of the four study sessions, but did not complete the full study due to scheduling obstacles. After obtaining baseline measurements, subjects were studied for four days at least one week apart. In random order, subjects receive all three treatments, or placebo versions of each treatment, under both stress and non-stress conditions. Neurocognitive and physical outcomes were measured.

## **Body**

All objectives identified in the original protocol statement of work were accomplished, although some took a little longer than initially anticipated, as described below.

## **REVIEW OF STATEMENT OF WORK OBJECTIVES**

### **• YEAR 1**

1. Institutional Review Board approval was obtained from Uniformed Services University, Bethesda, MD; the Human Subjects Research Review Board, Office of the Surgeon General, Department of the Army; the Scientific Planning and Review Committee, and the Committee for the Protection of Human Subjects, Naval Health Research Center, San Diego, CA; and the Bureau of Medicine, Department of the Navy, Washington, DC. Annual review with continuing approval was obtained from the USU IRB and the CPHS at NHRC, throughout the study period.
2. Two face-to-face meetings of key personnel took place early in the study, one in Bethesda and one in San Diego, to ensure that both sites were fully compatible and able to carry out the protocol in parallel with no difference in methods. Dr. Roy subsequently made periodic visits (every 6-12 months) to NHRC to ensure comparability of the two study sites. Quality Assurance specialists representing the study sponsor at USAMMDA, Fort Detrick, MD, also made regular visits to both study sites.

3. The HP gas chromatograph mass spectrometer was purchase at the start of the study and performed well throughout the study period, proving useful in validating new methods of DEET and permethrin in the plasma.
  4. Study personnel were successfully hired and retained at each site in order to carry out the study.
  5. Permethrin was obtained from the Department of Defense Supply Depot in Philadelphia, PA. Military battle dress uniforms (BDUs) were treated with permethrin by Dr. Roy following the IDA Kit methods described by the Armed Forces Pest Management Board in Technical Information Memorandum No. 36.
  6. Pyridostigmine bromide 30 mg tablets and DEET 33% cream (2 oz tubes) were obtained from the Department of Defense Supply Depot in Philadelphia, PA. Corresponding placebo tablets identical to the PB were obtained from the manufacturer, ICN, in Quebec, Canada. Identical-appearing 2-oz DEET tubes were prepared by 3-M, St. Paul, MN, where they were filled with a placebo cream, Cavilon, that had a similar appearance and texture to the DEET-containing cream.
  7. A pilot study of 10 subjects at USUHS established that DEET and permethrin could be effectively blinded for, in addition to identifying that the DEET could be detected in plasma when used appropriately, whereas permethrin could not.
  8. Upon completion of the pilot study, the main study was initiated, and subjects were enrolled at each site, completing the study as designed.
  9. Study monitors at each site were identified to be responsible for evaluation of individual patients who developed symptoms or illness during the study.
  10. An independent Data Safety and Monitoring Board (DSMB) was constituted to review study findings, to which investigators are blinded, to ensure that subjects were not harmed by participation in the study. The DSMB reviewed accrued data, including study-wide adverse events, at 3 to 6 month intervals. Although procedures were in place for the DSMB to notify the IRBs if interim findings from this study or other studies warranted early termination of this study, this was not required.
- **YEAR 2**
    1. Subject participation continued uneventfully.
    2. The study monitors and DSMB continued the duties outlined in Year 1.
1. **YEAR 3**
    1. Full study participation was completed by the targeted number of participants.
    2. The study monitor and DSMB continued to perform their duties successfully until subject participation was complete.
    3. After all subjects completed the protocol and an analysis of the complete data set was performed, investigators were unblinded to the results.

4. The findings were presented at several national scientific meetings, and publications have been submitted to peer-reviewed journals.
5. No significant adverse health effects of participation in the study were identified, so that while the Department of Defense was prepared to provide medical evaluation and treatment as indicated, this provision was not necessary.

## Key Research Accomplishments

- Established and validated highly sensitive methods of plasma analysis for DEET and permethrin, which has now been published.
- Demonstrated that permethrin applied to Battle Dress Uniforms is not absorbed into the bloodstream in detectable levels, even with the use of exquisitely sensitive assays.
- Demonstrated that combined treatment with PB, DEET, and permethrin, with short-term use as indicated, does not appear to adversely impact physical or cognitive performance, regardless of the presence of short-term multimodal stress.
- Study participants represented a broad sample of ethnicity, race, and gender, with deliberate over-sampling of women as compared with a typical active duty military population, as documented in Table 1, below.

**TABLE 1. Demographics of Subjects that Completed One or More Exposures**

Adults	American Indian or Alaska Native	Black or African American	Hispanic or Latino	Asian	White or Caucasian	Other	Total Male/ Female	Overall Percentages
Male	0	9	4	4	37	0	54	67%
Female	1	1	3	1	21	0	27	33%
Total	1	10	7	5	58	0	81	100%

## Reportable Outcomes

### Publications

1. Roy MJ, Kraus PL, Paton JC, Seegers CA, Coll R, Cherstniakova S, Koslowe PA, Law WA, Krantz DS, Deuster PA, Cantilena L. DEET and permethrin in stress conditions. *Military Med* (in press, scheduled for January, 2006).
2. Cherstniakova SA, Garcia GE, Strong J, Daoquin B, Weitz J, Roy MJ, Cantilena LR. Rapid determination of n,n-diethyl-m-toluamide and permethrin by gas chromatography-mass spectrometry and pyridostigmine bromide by high-performance liquid chromatography in human plasma. *J Analytical Toxicol* (in press).
3. Roy MJ, Kraus PL, Seegers CA, Young SYN, Kamens DA, Law WA, Cherstniakova S, Chang D, Cooper JA, Sato P, Matulich W, Krantz DS, Cantilena L, Deuster PA. Pyridostigmine, DEET, permethrin, and stress: A double-blind, randomized, placebo-controlled trial to assess harm. *Lancet* (in submission).

### Abstract Presentations

1. Roy MJ, Coll R, Seegers CA, Deuster PA, Barnes S, Cherstniakova S, Koslowe PA, Law WA, Krantz DS, Cantilena L, Goldstein RE. Randomized, Placebo-Controlled Study of DEET and Permethrin Under Stress Conditions. American College of Physicians-American Society of Internal Medicine, Army Region Annual Meeting, San Antonio, TX, November, 2001.
2. S. Cherstniakova, PhD, G. Garcia, PhD, J. Strong, PhD, N. Helbling, D. Bi, MD, M. Roy, MD, L.R. Cantilena, MD, PhD, Uniform Services Univ, Bethesda MD, WRAIR, Silver Spring, MD, CDER/FDA, Rockville, MD. Simultaneous Determination Of N,N-Diethyl-M-Toluamide And Permethrin By Gc-MS And Pyridostigmine Bromide By HPLC In Human Plasma. Application To Pharmacokinetic Studies. American Society for Clinical Pharmacology and Therapeutics 104<sup>th</sup> Annual Meeting. Washington, DC, April, 2003.
3. Roy MJ, Seegers CA, Deuster PA, Law WA, Young S, Sato P, Kamens D, Koslowe PA, Cherstniakova SA, Cantilena L. Randomized, Placebo-Controlled Trial of Combination Preventive Treatment with Pyridostigmine, DEET, and Permethrin. Society of General Internal Medicine 26<sup>th</sup> Annual Meeting, Vancouver, BC, May, 2003.
4. Roy MJ, Seegers CA, Deuster PA, Law WA, Young S, Sato P, Kamens D, Koslowe PA, Cherstniakova SA, Cantilena L. Randomized, Placebo-Controlled Trial of Combination Preventive Treatment With Pyridostigmine, DEET, And Permethrin. USU Research Day, Bethesda, MD, May, 2003.
5. Ashburn JR, Law WA, Roy MJ, Kim SJ, Seegers C, Deuster PA. The Relationship between Social Discomfort and Executive Functioning Poster Presentation, USU Research Day, Bethesda, MD, May, 2003.
6. Williams PCM, Johnson TE, Clark K, Saunders LL, Stutler SA, Winston GCH, Randolph DQ, Keller C, Roy MJ, Neal T. Correlating in vivo and in vitro models: bioeffects for



safety standards of human exposure to 3.8 micron lasers. Fifth Annual Non-lethal Technology and Academic Symposium. Arlington, VA, November, 2003.

7. Kamens DR, Young SN, Ryan MK, Cantilena L, Deuster PA, Law WA, Matulich WJ, Paton JC, Kraus PL, Seegers CA, Koslowe PA, Cherstniakova SA, Roy MJ. Assessing the safety of combination treatment with Pyridostigmine, DEET, and Permethrin. The Forty-Third Navy Occupational Health and Preventive Medicine Workshop, Virginia Beach, VA, March, 2004.
8. Kraus PL, Roy MJ, Paton JC, Young SYN, Deuster PA, Law WA, Seegers CA, Cherstniakova SA, Krantz DS, Cantilena L. Randomized, placebo-controlled trial of combination preventive treatment with pyridostigmine, DEET and permethrin. USU Research Day, Bethesda, MD, May, 2004.
9. Kim S, Law WA, Ashburn JA, Deuster PA, Roy MJ. Neurocognitive features of ADHD in a non-clinical adult sample: Do people with ADHD symptoms have similar cognitive weaknesses seen in ADHD patients? Poster Presentation, USU Research Day, Bethesda, MD, May, 2004.
10. Roy MJ, Young SYN, Deuster PA, Law WA, Seegers CA, Kamens DR, Kraus PL, Paton JC, Cherstniakova SA, Krantz DS, Cantilena L. Peer-Reviewed Medical Research Programs Military Health Research Forum. San Juan, PR, April, 2004.
11. Roy MJ, Young SYN, Deuster PA, Law WA, Seegers CA, Kamens DR, Kraus PL, Paton JC, Cherstniakova SA, Krantz DS, Cantilena L. PB, DEET and permethrin: A randomized controlled trial to assess safety. 2004 Bioscience Review, Hunt Valley, MD, May, 2004.
12. Roy MJ, Young SYN, Deuster PA, Law WA, Seegers CA, Kamens DR, Kraus PL, Paton JC, Cherstniakova SA, Krantz DS, Cantilena L. Randomized, placebo-controlled trial of combination preventive treatment with pyridostigmine, DEET, and permethrin. 7<sup>th</sup> Annual Force Health Protection Conference. Albuquerque, NM. August, 2004.
13. Roy MJ, Young SYN, Deuster PA, Law WA, Seegers CA, Kamens DR, Kraus PL, Paton JC, Cherstniakova SA, Krantz DS, Cantilena L. Randomized, placebo-controlled trial of combination preventive treatment with pyridostigmine, DEET, and permethrin. XXXV International Congress on Military Medicine. Arlington, VA, September, 2004.
14. Roy MJ, Young SYN, Deuster PA, Law WA, Seegers CA, Kamens DR, Kraus PL, Paton JC, Cherstniakova SA, Krantz DS, Cantilena L. Randomized, placebo-controlled trial of combination preventive treatment with pyridostigmine, DEET, and permethrin. 21<sup>st</sup> Annual Army American College of Physicians Meeting, Arlington, VA. November, 2004.

## Conclusions

Salient results of this prospective, randomized controlled trial include the following:

1. Pyridostigmine bromide (PB), DEET, and permethrin can be rapidly and sensitively assayed in human plasma samples.
2. Permethrin applied to Battle Dress Uniforms does not enter the bloodstream at detectable levels, whereas DEET applied to the skin, and PB ingested orally, are readily detectable. PB levels are generally consistent with previously published pharmacokinetic patterns, but it was found that higher bloodstream PB concentrations occurred under stress conditions than at rest, persisting up to one hour after stress, but returning to non-stress levels within 3 hours after stress. Nevertheless, elevated PB concentrations were not found to influence neurocognitive or physical performance.
3. Systolic blood pressure and heart rate significantly increased with stress, whereas diastolic pressure did not. There were no differences in hemodynamic measures when subjects were exposed to the combination of PB, DEET, and permethrin, as compared to placebos.
4. Neurocognitive outcomes were measured using the S-CAT computerized battery developed by NASA. Short-term combined physical and mental stress resulted in improved performance on subsequent the S-CAT, regardless of exposure to the combination of PB, DEET, or permethrin, or corresponding placebos. Neither stress nor exposure to the combination of PB, DEET and permethrin, influenced subsequent physical performance, including handgrip strength and duration, timed stair-climbing, pushups (females only) or pull-ups (males only).
5. The combination of PB, DEET, and permethrin was well-tolerated by participants, with all reported symptoms occurring at least as often, or more so, with placebos than with active treatments.

## **Appendices**

- I. Manuscript in press at Military Medicine: Initial Evaluation of DEET and Permethrin Absorption in Human Volunteers Under Stress Conditions, page 13
- II. Manuscript under review at Lancet: Pyridostigmine, DEET, permethrin, and stress: A double-blind, randomized, placebo-controlled trial to assess harm, page 32

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## **Initial Evaluation of DEET and Permethrin Absorption in Human Volunteers Under Stress Conditions**

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## ABSTRACT

**Objectives:** This is a pilot study to determine whether: 1) it is feasible to effectively blind human subjects to the presence of the insect repellents DEET and permethrin; 2) whether DEET affects the absorption of permethrin; and 3) whether combat videos viewing and mental arithmetic are stressful.

**Methods:** Ten volunteers were exposed to DEET, permethrin and stress (one-hour combat video plus mental arithmetic) in a double-blind, randomized, placebo-controlled trial. Outcome measurements included hemodynamics, plasma DEET and permethrin levels, and questionnaires to assess blinding.

**Results:** Highly sensitive serologic assays readily detected DEET, but not permethrin. Staff and subjects were effectively blinded to both. The video-math combination was stressful by both self-report and hemodynamic measures.

**Conclusions:** It is possible to blind for DEET and permethrin. Permethrin on clothing does not enter the bloodstream at appreciable levels. Combat videos and mental arithmetic can be stressful.

## Introduction

The insect repellents *N,N*-diethyl-*m*-toluamide (DEET) and permethrin are widely used by the U.S. military, and both were distributed in limited quantities during the 1990-91 Persian Gulf War. Permethrin was available to some soldiers, either in 6 oz cans as a 0.5% aerosol form, or in individual duty application (IDA) kits, for application to Battle Dress Uniforms (BDUs). It is estimated that perhaps 2% of those deployed for Operations Desert Shield and Desert Storm treated their BDUs with permethrin. DEET was more widely available, with an average of 2.6 2-ounce tubes distributed to each deployed soldier.<sup>1</sup> Both repellents are available commercially and widely used in adults and children in various formulations. While excessive exposure can be harmful, use as indicated appears to be quite safe.<sup>2,3</sup>

If enemy use of nerve agents is threatened, the carbamate pyridostigmine bromide (PB) provides protection by reversibly inhibiting 20-40% of neuromuscular junction acetylcholinesterase (AChE), blocking irreversible binding by nerve agents. Whereas animal and human studies have demonstrated safe, independent use of PB, DEET, and permethrin, some investigators implicate the combination in a variety of ill-defined symptoms reported by some Gulf War veterans.<sup>4</sup> Abou-Donia *et al.*<sup>5</sup> reported neurotoxic effects in chickens subjected to the equivalent of a 70 kg soldier taking 467 PB pills, 76 tubes of DEET and 1,667 cans of permethrin daily. This compares with an average of less than 3 tubes of DEET and 2 cans of permethrin per soldier *available* during the entire Gulf War.<sup>2</sup> In another study PB, DEET, and permethrin were administered orally to rats, and the toxicity was less than expected, with no evidence of synergy between DEET and permethrin, despite oral administration. However, the investigators found a high mortality rate with the addition of very high doses of PB to the DEET and permethrin.<sup>6</sup> Abou-Donia *et al.* concluded that there is a need for "additional studies into potential health risks associated with co-exposure of humans to these agents at dosages likely to have been used by Gulf War veterans."<sup>7</sup> We concur with this conclusion, but note that Abou-

Donia's subsequent studies continue to involve excessive doses and/or inappropriate routes of exposures in laboratory animals.<sup>8,9</sup>

In preparation for a multi-center, double blind, randomized, placebo-controlled crossover trial involving administration of DEET, permethrin and PB to 64 healthy young volunteers under stress and rest conditions, we performed a pilot study with 10 subjects. The purposes of the pilot study were (1) to validate that simultaneously combat video viewing and mental arithmetic is stressful; (2) to determine whether study staff and participants can be effectively blinded to the presence of DEET and permethrin; (3) to determine whether DEET and permethrin can be detected in plasma when used in accord with military doctrine, and (4) if so, whether DEET blocks absorption of permethrin (as had been seen with rodent and pig skin *in vitro*).<sup>10</sup>

## **Methods**

### **Participants and Setting**

We recruited 10 young, physically fit individuals to participate in this study in the Human Performance Laboratory (HPL) at Uniformed Services University of the Health Sciences (USUHS), Bethesda, MD. Individuals 18 to 49 years of age were eligible for the study. Exclusion criteria included a history of known coronary artery disease; hypertension; diabetes mellitus; morbid obesity; osteoarthritis, or other chronic joint, muscle, or nervous system disorder; active, widespread skin conditions including but not limited to eczema and psoriasis; and significant allergic reactions to DEET, permethrin, pyridostigmine, or similar compounds. A board-certified internist performed a medical history and physical exam on participants to confirm good general health. The USUHS Institutional Review Board Human Use Committee approved the study and all participants signed an approved Informed Consent Document prior to participation.

## **Design**

The study was a double blind, randomized placebo-controlled crossover trial that exposed participants to chemicals or corresponding placebos, in conjunction with viewing war videos and solving math problems on two different occasions separated by approximately one week. On one of the two occasions, participants wore permethrin-impregnated uniforms and applied DEET-containing cream to their skin, while on the other occasion they applied a placebo cream, and wore uniforms that may or may not (50% in each category) have been treated with permethrin (Table 1). As a result, DEET-containing cream was applied in 10 of 20 total study sessions, whereas permethrin-treated uniforms were worn in 15 of 20 total sessions; placebos were employed in the remainder. Both participants and investigators were blinded to the presence of DEET and permethrin.

## **Procedure**

For each study session, participants arrived at the HPL between 0730 and 0800. Weight, height, blood pressure, and heart rate was recorded. Baseline blood samples were obtained to measure serum cortisol and plasma DEET and permethrin levels at 0800. Participants donned BDUs and applied DEET or placebo cream to their forearms, neck, and face. They were then at liberty to go to work or class (most participants were USUHS employees or medical students), and instructed to return to the HPL at 1215 that afternoon for the placement of an intravenous catheter in one forearm. The site of catheter placement was carefully cleaned in advance with alcohol swabs to remove any cream from the immediate area, and was then covered with a clear adhesive wrap (OpSite FLEXIGRID Transparent Adhesive Film Dressing, Smith & Nephew, Inc., London, UK) followed by 1" Coban (3M, St. Paul, MN) self adhesive wrap to avoid recontamination. Blood was drawn from the catheter for cortisol, DEET, and permethrin levels at 1255, with repeat levels at 1330 and 1400, halfway through and at the end of the video exposure session, respectively. Participants were seated 10 feet away from a large video screen



and were instructed to direct their attention to the video screen at all times. The 60-minute video was initiated at 1300; after the 1330 blood draw, 6 60-second mental arithmetic sessions were initiated at defined intervals (Figure 1) in order to assess the degree and duration of impact of the mathematical problem solving upon hemodynamic measures. Blood pressure and heart rate were measured every 5 minutes throughout the video viewing period using an automated blood pressure cuff (Criticare Systems, Inc., Waukesha, WI).

Upon completion of each 60-minute video session, participants completed a questionnaire assessing whether they believed they were exposed to DEET and/or permethrin, and how sure they were about either. They were also asked how stressful they thought the video segments and math problems were. A blinded staff member who observed the full session completed a similar questionnaire.

## Exposures

Standard United States Army 100% cotton lightweight BDU blouses and trousers (Propper International®) were obtained in multiple sizes for the study. One-half of the uniforms were treated with permethrin using Individual Dynamic Absorption (IDA) Kits, NSN 6840-01-345-0237, provided by the Defense Logistics Agency, Richmond, VA. Treatment of the uniforms was performed by the primary investigator (MJR) in accord with procedures described by the U.S. Army Environmental Hygiene Agency.<sup>11</sup> Treated uniforms were stored and laundered separately to avoid contamination. One investigator alone maintained a randomization sheet to direct distribution of appropriate uniforms and creams. A different, blinded investigator completed questionnaires regarding exposures. Participants were given standard Army brown tee shirts to wear under the BDUs.

N,N-Diethyl-m-toluamide (DEET), 31.58% concentration (3M Company, St. Paul, MN), was also provided by the Defense Logistics Agency, Richmond, VA, in standard Army-issue olive-green 2-ounce tubes. Consultation with scientists at the 3M Company established that Cavilon™ Durable Barrier Cream Dimethicone Skin Protectant (Active Ingredient: Dimethicone 1.3%) as the product most closely resembling the DEET-containing cream in appearance and consistency. This was prepared as the placebo cream for the study in identical Army olive-green 2-ounce tubes. A study staff member dispensed approximately 2.5 grams of the appropriate cream directly from the tube into the hand of each participant on the morning of their participation, and observed the participant rubbing it into their skin.

Two one-hour videos were created by the primary investigator utilizing combat scenes compiled from seven war-related movies: *Saving Private Ryan*, *Platoon*, *Apocalypse Now*, *Full Metal Jacket*, *Hamburger Hill*, *All Quiet on the Western Front*, and *The Deer Hunter*.

Mathematical problems were administered in accord with methods previously validated and described.<sup>12,13</sup> Participants were told to subtract the number 7 serially from an initial 4-digit number (e.g., 1089). A study staff member provided participants with vigorous and frequent

prompts, such as "Faster", "Louder", and "Keep your eyes on the video screen", throughout each 60-second math period. Participants were aggressively informed of wrong answers and told to resume their subtraction from the previous correct number. Participants demonstrating tremendous difficulty with serial 7s were downgraded to serial 3s, while those who breezed through serial 7s were challenged with serial 13s and/or 17s.

### **Pharmacologic Procedures**

*A rapid and highly sensitive gas chromatography/mass spectrometry (GC-MS) method for simultaneous determination of N,N-Diethyl-m-toluamide (DEET) and permethrin was developed and validated. We have described this in detail elsewhere.<sup>14</sup>*

#### *Statistical Analysis*

SAS (SAS/STAT User's Guide Version 8, SAS Institute Inc., Cary, NC) was used for statistical data management and data analysis. To assess the efficacy of blinding the level of agreement between the observer (participant or staff member) assessment and the actual exposure was assessed using the simple kappa statistic,  $\kappa$ . The kappa coefficient is a measure of inter-rater agreement, quantifying the extent to which the observed agreement exceeds that which would have been expected by chance alone.<sup>15</sup> If there were complete agreement,  $\kappa = 1$ , whereas in a case of complete disagreement,  $\kappa = -1$ . A generalized linear model (GENMOD) was used in the analysis of repeated measures (eg., heart rate and blood pressure).

### **Outcome Measures**

Measures of stress included heart rate and blood pressure at 5-minute intervals throughout the 60-minute video exposure period, as well as serum cortisol levels at the 30- and 60-minute mark of the video exposure, compared to baseline. The efficacy of blinding staff and participants to the presence of DEET and permethrin was assessed via questionnaires. The potential for interaction between DEET and permethrin was assessed through the measurement of plasma levels at the 0-, 30-, and 60-minute marks of the video exposure session, with baseline absence of exposure confirmed by assays prior to exposure to the chemicals.

## Results

Five men and five women participants each completed two study sessions that were temporally separated by one week. Six were Caucasian, two were Latino, one was African American, and one was a Pacific Islander. The mean age of participants was 32, with a range from 23 to 41. There were no adverse effects identified during the course of the study.

Serum cortisol levels did not prove to be helpful in judging the stressfulness of this short-term exposure, as no correlation could be discerned between serum levels and the introduction of stress. Hemodynamic measurements did not indicate evidence of stress when participants were exposed to combat scenes alone during the first 30 minutes of the stress period, but did show significant increases in heart rate and systolic blood pressure after the math problems were added at the 30-minute mark (Figure 2). The most significant differences were present on the first hemodynamic readings after the initiation of math problems, at the 35-minute mark, and are denoted in the legends in Figures 2a and 2b. Diastolic blood pressure (Figure 2c) demonstrated a similar pattern during the stress period, but the changes were not statistically significant. Similar hemodynamic response patterns were seen with both videos.

Overall, the blinding of participants to DEET and permethrin was effective. In six of 10 sessions, the participants correctly identified exposure to DEET-containing cream, and 7 of 10 correctly identified exposure to the placebo cream. However, the number of correct responses did not significantly differ from what might have occurred based on chance alone ( $\kappa = 0.3$ ; 95% CI -0.116, 0.716). In addition, and perhaps of greater importance, the level of confidence among those who attempted to identify the presence or absence of the DEET-containing cream was weak. Only one participant was sure of the identity of the cream in each case; the other 5 identifying DEET correctly were either somewhat sure (2) or not at all sure (3), and the other 6 who correctly identified the placebo cream were likewise somewhat sure (3), had a hunch (1), or

were not at all sure. The participants who were incorrect all acknowledged hunches or were not at all sure. In 7 of 15 sessions, participants correctly identified permethrin-treated uniforms, and all five participants correctly identified the session in which they wore untreated uniforms. In this case, the ability of participants to identify the uniform treatment status was statistically significant, though the  $\kappa$  value was again 0.3 (95% CI 0.0361, 0.5726). However, here too, most of the responses were mere guesses at the status of the uniform: of the seven who correctly identified permethrin-treated uniforms, only one participant was very sure, and one was somewhat sure—most were either playing hunches (3) or not at all sure (2). Likewise, participants reported little certainty with their correct identification of the 5 untreated uniforms, being somewhat sure (1), a hunch (1), or not at all sure (3).

The efficacy of blinding the investigators was also assessed. One investigator who was blinded with respect to exposure status was asked to judge whether each of the participants was exposed to DEET or permethrin, and was unable to reliably do so ( $\kappa = 0.2$  for DEET, CI  $-0.19, 0.59$ ; and  $\kappa = 0.24$  for permethrin, CI  $-0.1, 0.58$ ), nearly always either based on a hunch or being not at all sure. Overall, the investigator correctly identified 4 of 10 treated with DEET, and 8 of 10 with placebo cream, as well as 8 of 15 permethrin-treated uniforms and 4 of 5 untreated uniforms.

Pharmacologic analyses of plasma samples for DEET and permethrin uniformly showed that permethrin was undetectable in the bloodstream for all participants, regardless of the presence of DEET-containing cream. DEET, on the other hand, was detected at levels logarithmically higher than expected in two participants exposed to DEET-containing cream. In addition, DEET was identified at low levels ( $< 10$  ng/ml) in two different participants who were only exposed to placebo cream. These findings strongly suggest that DEET contaminated the phlebotomy site or (less likely) the sample further along in the laboratory process. This was corroborated by swabbing and testing tourniquets, countertops, and other surfaces in the laboratory, which were found to have DEET even after routine cleaning procedures.

Comparison of a variety of cleaning methods established that the most effective method of decontamination of laboratory surfaces is to clean with Formula 409® cleaner, followed by soap and water. To eliminate another potential source of contamination, the placebo cream was tested, confirming that it did not in fact contain any DEET.

## Discussion

The results of this randomized controlled trial are significant for both the conduct of our subsequent study involving similar exposures in conjunction with the nerve agent prophylaxis, pyridostigmine bromide, as well as for other studies and military medicine in general. First, we demonstrated that it is possible to blind most individuals to the presence of DEET and permethrin, with some caveats. Whereas most participants could not confidently discern DEET-containing cream from placebo cream, there were two participants with significant military deployment histories involving frequent use of DEET, for whom the distinctive odor of DEET was a reliable discriminator. However, nearly all of our participants were active duty service members who had some experience with DEET, and most were not able to identify the DEET-containing cream.

With regard to the permethrin-treated BDUs, we noted that after the initial treatment of the uniforms, a visible residue was present on some areas of the fabric. This enabled our first two participants to be relatively certain of the treatment status of the uniforms. After the first two participants, however, we laundered all the uniforms, and the remaining participants had no way to distinguish treated from untreated uniforms. It should be noted that after permethrin application using the IDA kit, the uniforms can be laundered—though not dry-cleaned with chlorofluorocarbon-based solvents (CFCs)—without losing the permethrin impregnation.<sup>11</sup> It was important for us to be able to establish a reasonable degree of blinding to maintain the integrity of the design of our subsequent study.

The second issue we attempted to address in this study was to examine potential interactions between DEET and permethrin. Studies of rodent and porcine skin *in vitro* suggested that DEET inhibited the absorption of permethrin.<sup>10</sup> Our study design was intended to facilitate the comparison of plasma permethrin levels with and without concomitant DEET exposure. However, permethrin was never detectable in the plasma regardless of the presence of

DEET. This did not allow us to determine whether there are interactions between DEET and permethrin in humans *in vivo*. Nevertheless, the inability to detect permethrin, despite a very sensitive assay, is important, since it suggests that soldiers wearing permethrin-treated uniforms do not have significant exposure. This is not surprising, since permethrin is only on the outside of the uniform, which would not really be expected to result in significant exposure. Furthermore, it suggests that studies in which animals are exposed to very high levels of permethrin have limited generalizability to military service members. Although this study involved a relatively short-term exposure, with participants wearing treated uniforms for a total of only 6 hours, our subsequent study will involve 30 hours of exposure. Longer exposure times will provide more information, but this short-term exposure already begins to point out potentially significant flaws in previous animal studies. Proper use of permethrin is generally considered to be quite safe, even in children who are still developing neurologically. In fact, its significant safety advantage over its predecessor, lindane, has led to permethrin becoming the treatment of choice for lice. However, at high doses, permethrin may have the potential for neurologic and reproductive system toxicity. Abou-Donia et al.<sup>5,7-9</sup> exposed animals to very high levels of DEET permethrin, either through subcutaneous injection or direct dermal application at a site close to the brain (posterior neck), and the resulting toxicity may be directly related to the permethrin exposure, which our results suggest far exceeds the degree of exposure of deployed military service members. There is also evidence that avian species are more sensitive to insecticides than mammals, and evidence that rats absorb DEET more readily than do humans.<sup>3,16-17</sup>

The final purpose of this study was to determine whether combat video footage in conjunction with mathematical problem solving is stressful. We found that while cortisol levels did not evidence a consistent response to this short-term stress, hemodynamic measurements did manifest a consistent, significant pattern of response. When combat videos were shown without

concomitant math problems, no significant changes in heart rate or blood pressure were noted. However, when math problems were superimposed, we did see significant increases in both heart rate and systolic blood pressure. The increases were transient, being most pronounced with the initial math session, and when vital signs were measured immediately following the math session. Study participants and staff members also judged the math element to be more stressful than the video element, although a prisoner-of-war scene from the movie *The Deer Hunter* was described as stressful by many participants, even if they had previously seen the movie. These results corroborate previous studies that have demonstrated cardiovascular stress associated with similar mathematical problem solving. The math sessions we used in this pilot study were shorter in duration than in previous studies, and longer math sessions may result in a longer duration of effect. War, especially on the modern battlefield, involves more than simply physical stress, with issues of judgment and calculations required of many service members resulting in superimposed neurocognitive stress. The math problems provide an approximation of this type of stress.

The most notable limitation of our study is that it evaluates short-term exposures and short-term stresses. It may be that prolonged exposure to permethrin-treated uniforms would result in some absorption, although it is still likely to be far less intense and significant than subcutaneous injection or direct dermal application of relatively high concentrations of permethrin that have been employed in animal studies. With regard to the stressors employed in this study, we were successful in demonstrating that the math element resulted in hemodynamic stress, but this short-term stress may not have the same physiologic effect as more chronic stress. Another limitation in this pilot study is our small sample size. We did sample size analyses for our full study, but did not do so for this pilot segment, which renders our conclusions with regard to the efficacy of blinding to the presence of DEET less robust.

Despite efforts to protect the phlebotomy site in study participants, we identified contamination of plasma samples, which may be attributable to one or more factors: insufficient cleaning of the forearm prior to placement of an intravenous heptlock; participants touching cream-laden areas and then touching their uniform or the tape or bandage over the intravenous heptlock, which was subsequently handled by the phlebotomist; diaphoresis of participants leading to diffusion of cream; and contamination of the tourniquet, with subsequent contamination of the sample by the phlebotomist who handled the tourniquet. This led to several important modifications to our subsequent protocol to avoid, or at least limit, contamination: application of cream to the legs rather than the forearms; use of gloves after application; disposal of the tourniquet after each use; and more thorough cleaning of all laboratory surfaces after each participant session.

In summary, this pilot study provides important implications for our subsequent, much larger randomized controlled trial, in establishing the efficacy of our assays for DEET and permethrin, and in documenting that the video-math combination is stressful without significant differences between the two one-hour videotapes. The fact that the permethrin could not be detected in the plasma of any participant also is particularly important in beginning to point out potential problems with the generalizability of animal studies that have purported to show synergistic toxicity of high doses of pyridostigmine bromide, DEET, and permethrin. Our full study, currently in progress, should enable us to comment at greater length on this subject.



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Table 1: Randomization Scheme

SUBJECT	TRIAL 1		TRIAL 2		DEET	VIDEO
	PERMETHRIN/ NUMBER		PERMETHRIN/ DEET	VIDEO		
1	PE/DEET	A	PE/PL	B		
2	PL/PL	A	PE/DEET	B		
3	PE/DEET	B	PE/PL	A		
4	PE/PL	A	PE/DEET	B		
5	PL/PL	B	PE/DEET	A		
6	PE/DEET	A	PL/PL	B		
7	PE/PL	B	PE/DEET	A		
8	PL/PL	B	PE/DEET	A		
9	PE/DEET	B	PL/PL	A		
10	PE/DEET	A	PE/PL	B		

(PE = permethrin; PL = placebo)

## **Legends for Figures**

**Figure 1: Timing of hemodynamic measurements and math sessions**

**Figure 2.a: Heart Changes During Stress**

**Figure 2.b: Systolic Blood Pressure During Stress**

**Figure 2.c: Diastolic Blood Pressure During Stress**

Pyridostigmine, DEET, permethrin, and stress: A double-blind, randomized, placebo-controlled trial to assess harm

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## ABSTRACT

**Background:** Years after the Gulf War, many veterans have persistent symptoms. Animal studies suggesting synergistic toxicity of high-doses of the nerve agent prophylactic pyridostigmine bromide (PB) and insect repellents DEET and permethrin, especially under stress, have led to hypotheses of causation in Gulf War veterans.

**Objective:** To determine whether human exposure to PB, DEET, and permethrin, under stress, adversely impacts short-term physical or neurocognitive performance.

**Design:** Prospective double blind, randomized, placebo-controlled, crossover trial.

**Setting:** Military research facilities in Bethesda, MD, and San Diego, CA.

**Participants:** Eighty-one healthy men and women ages 21 to 49.

**Intervention:** Permethrin-impregnated uniforms, DEET-containing skin cream, oral PB, and corresponding placebos. Participants had 4 separate sessions, ensuring exposure to all treatments and placebos under both stress and rest conditions in variable order.

**Measurements:** Physical performance was assessed by handgrip strength and duration, stair climbing, and pull-ups (males) or push-ups (females). Neurocognitive performance was assessed by the validated computer-based WinSCAT. Side effects were reported by participants in response to open-ended questions.

**Results:** Permethrin was undetectable in the serum of all subjects; PB levels were higher immediately after stress (41.6 ng/ml; 95% CIs: 35.1, 48.1) than rest (23.0 ng/ml; 95% CIs: 19.2, 26.9), whereas DEET levels did not significantly differ by stress condition. Heart rate and systolic blood pressure increased significantly with stress compared to rest, but did not vary with treatment versus placebo. Physical and neurocognitive outcome measures, and self-reported side effects, did not significantly differ by exposure group.

**Limitations:** Short-term exposure and stress, with short-term outcome measures in healthy participants.



Conclusions: Combined, correct use of PB, DEET, and permethin is well-tolerated and without evidence of short-term physical or neurocognitive impairment.

## INTRODUCTION

Nerve agents can be easily disseminated to rapidly induce respiratory arrest and death, which renders them likely agents of terrorism or war. Post-exposure antidotes may be difficult to administer quickly enough to prevent nerve agent-induced aging of the target enzyme acetylcholinesterase (AChE)—especially for soman, which induces aging within 2 minutes.<sup>1</sup> Pyridostigmine bromide (PB), a quaternary carbamate that reversibly binds AChE at the neuromuscular junction, protecting AChE against irreversible binding by nerve agents, was employed prophylactically by American and British soldiers in the 1990-91 Gulf War.

The Food and Drug Administration (FDA) approved PB for myasthenia gravis therapy in 1955 at far greater doses than the 30 mg thrice-daily prophylactic dose.<sup>1,2</sup> During the Gulf War, roughly 50% of soldiers reported PB side effects, but only 1% sought medical attention, and less than 0.1% needed to stop the medication.<sup>2</sup> Subsequent research found short-term PB was well tolerated under heat stress conditions,<sup>3</sup> and did not impair acceleration tolerance,<sup>4,5</sup> driving ability or psychomotor skills,<sup>6</sup> neuromuscular function,<sup>7</sup> or bronchial hyperreactivity.<sup>8</sup> It is improbable, but not impossible, for a drug with a 2-3 hour serum half-life to induce symptoms years later, yet some implicate PB in the controversial “Gulf War Syndrome”, noting acetylcholine’s role in memory, sleep, and pain.<sup>9</sup>

As a quaternary amine, pyridostigmine should not significantly penetrate the blood-brain barrier; there is conflicting evidence that stress might facilitate penetration.<sup>10-12</sup> Though some Gulf War veterans (GWVs) reported neurocognitive complaints, such as memory or concentration difficulties, a link to PB is lacking.<sup>13</sup> GWVs acknowledge more symptoms than controls,<sup>14,15</sup> but self-reported symptoms are subject to multiple biases. Similar symptoms have been reported after other wars,<sup>16</sup> when PB was not used.

Some indict the combination of PB with the insect repellents, *N,N*-diethyl-*m*-toluamide (DEET) and permethrin, which had limited distribution during the Gulf War. It is estimated that

2% of U.S. soldiers treated their Battle Dress Uniforms (BDUs) with permethrin. Less than three 2-ounce tubes of 33% DEET were distributed per soldier; although data are lacking on actual use, soldiers rarely employed it while taking PB, since insects were then uncommon.<sup>17</sup> Whereas excessive repellent exposure can be harmful, proper independent use appears quite safe.<sup>18,19</sup> Self-reported repellent use was associated with persistent symptoms in one study of GWVs,<sup>20</sup> and a study in hens<sup>21</sup> reported neurotoxic effects with per kilogram exposures hundreds of times greater than that of GWVs.<sup>17</sup> Another study found rats tolerated oral DEET and permethrin well, but suffered high mortality with addition of high dose PB.<sup>22</sup> After another high-dose animal study, investigators cited a need for "additional studies into potential health risks associated with co-exposure of humans to these agents at dosages likely to have been used by Gulf War veterans."<sup>23</sup>

The primary purpose of this study was to determine whether appropriate dosage and administration of PB, DEET, and permethrin, during stressful and non-stressful conditions, impact immediate human physical and/or neurocognitive performance.

## **METHODS:**

### **Participants**

We recruited healthy individuals (Table 1) ages 18 to 49, who were beneficiaries of the Department of Defense Healthcare System (active duty service members, retirees, or their dependents). All participants signed an Informed Consent Document. The study design and consent form were approved by human use committees at the Uniformed Services University (USU), Bethesda, MD, Naval Health Research Center (NHRC), San Diego, CA, Office of the Surgeon General of the Army, and Navy Bureau of Medicine and Surgery. Exclusion criteria were coronary artery disease; hypertension; diabetes mellitus; morbid obesity; any chronic joint, muscle, or nervous system disorder; a widespread skin condition such as eczema or psoriasis; or a history of an allergy to DEET, permethrin, pyridostigmine, or similar products. Health status

was confirmed by a medical history, physical examination, electrocardiogram, and screening laboratory studies. Participants provided demographic information including self-identified race, sex, age, and military rank, and completed baseline questionnaires including the PRIME-MD Today®, SF-36 measure of functional status, and Posttraumatic Stress Disorder Checklist-Civilian Version (PCL-C). The study population was intended to represent the active duty military, except for over sampling women to assess potential gender differences. Participation occurred on a rolling basis from August 2001 through December 2003. The design allowed only one participant per day, and was further dependent upon nursing staff availability, so 17 participants who completed at least one session were deployed prior to study completion (Figure 1); however, the target number of 64 completed the full study, and no subjects were lost to follow-up.

## **Design**

A randomized placebo-controlled trial was conducted at USU and NHRC, exposing healthy participants to permethrin-impregnated uniforms, DEET-containing skin cream, and oral PB, in a manner consistent with U.S. military doctrine. A crossover design required participation on 4 different occasions, separated by at least 5 days, ensuring exposure to all treatments and placebos under both stress and rest conditions in an order determined via block randomization. Participants, investigators, and outcomes evaluators were blinded to treatment status.

## **Treatments**

***For each study period, participants were admitted to a supervised clinical research unit by 0800, 24 hours prior to their scheduled one-hour stress or rest session. An intravenous catheter was inserted to facilitate blood draws.***

An independent pharmacy employee maintained study medications in locked, temperature-controlled conditions, and distributed 30 mg PB, or matching placebo, tablets (ICN, Quebec, Canada) to staff prior to each study period. A blinded study nurse administered one tablet each at 0800, 1600, and 2400 on the first day of each study period, and again at 0800 (immediately prior to the one-hour stress or rest session) the following morning. The pharmacy

employee also distributed .25 grams of DEET (33%, prepared for the Department of Defense by 3M Company, St. Paul, MN) or corresponding placebo cream (Cavilon, 3M Health Care, St. Paul, MN—active ingredient, dimethicone, 1.3%) in clear plastic containers. At 0800 and 2000 on the first day, and again at 0800 the following morning, gloved participants evenly applied cream to the neck, face, and both legs, between the knee and the ankle (application was to the legs because DEET contaminated blood draws in a pilot study with arm application).<sup>24</sup>

Participants wore standard lightweight BDU blouses and trousers (Propper International®) for the entirety of each study period. Half the uniform sets were treated with permethrin using IDA Kits (NSN 6840-01-345-0237), in accord with U.S. Army protocol.<sup>25</sup> The pharmacy employee stored and distributed BDUs before each study period, and separately laundered them afterward.

### ***Physical and Mental Stress Sessions***

A one-hour march was conducted on a motorized treadmill (Quinton Medtrack ST65, Quinton Instruments, Bothell, WA). A backpack was secured on the participant, containing 27% (females) or 30% (males) of the participant's total body weight. (Initially, females had the same parameters as males, but after the first 3 females at NHRC—though not at USU—were unable to complete the 60-minute stress period, regardless of treatment or placebo exposure, modifications were made based on the difference in percent lean body mass between males and females, and all females subsequently completed all study sessions.) The test began with a 2-minute warm-up at 3.0 mph and 2.0% grade, and advanced to 3.5 mph and 4.0 % (females) or 5.0% grade (males) for the hour. Throughout the hour, participants viewed combat movie scenes on a large screen directly before them. In addition, participants performed mental arithmetic for six 90-second periods at 9-minute intervals, beginning with subtraction of serial 7's from a 4-digit number (e.g., 1089), a procedure known to increase blood pressure, heart rate, catecholamine levels, and self-reported stress.<sup>26</sup> Depending upon their mathematical proficiency, some participants were

subsequently switched to serial 3's, 13's, or 17's, to try to maintain consistent stress levels. Study staff aggressively corrected wrong answers. Heart rate (Polar Accurex Plus, Polar Electro Inc., Woodbury, NY) and blood pressure (manual) were recorded at baseline and at 3 and 9-minute intervals, respectively, during sessions. Blood was drawn and vital signs were repeated at the conclusion of the hour.

### ***Rest Sessions***

Participants read quietly in a chair for one hour, while blood draws and hemodynamic measurements mimicked the stress session schedule.

### ***Outcome Measures***

#### **Neurocognitive Battery**

Neurocognitive outcomes were measured immediately after all sessions using a validated, computerized performance assessment battery, the NASA-1 Spaceflight Cognitive Assessment Tool for Windows, or WinSCAT. The National Aeronautics and Space Administration adapted the WinSCAT from the Automated Neuropsychological Assessment Metrics battery for spaceflight cognitive assessments.<sup>27</sup> The WinSCAT requires dichotomous responses utilizing adjacent mouse buttons, and is valid for repeated measures applications. It takes approximately 15 minutes to complete, evaluating such factors as reaction time, attention span, and short-term memory. Performance is scored in five categories—code substitution, code memory (long delayed recall), continuous running memory, match to sample, and mathematical processing. Overall performance is assessed by incorporating the speed and accuracy of responses with an average WinSCAT outcome score for each exposure session, in turn derived by standardizing scores for each task referent to baselines. Prior to exposure sessions, participants completed six practice runs to establish baseline consistency and to minimize a learning effect in exposure sessions.

### **Physical Performance Battery**

Participants completed two practice sessions to establish proficiency on a battery developed and validated at the Naval Medical Research Institute, Bethesda, MD, to measure strength, endurance, and coordination. The battery features: 1) maximal voluntary contraction (MVC) with a handgrip dynamometer followed by measurement of the maximum duration of isometric contraction at 30% of the MVC (computer integration of strength and duration then provided a third handgrip measure); 2) number of stairs stepped, up and down, in one minute while wearing a 20 kg weight belt; 3) number of pull-ups (males) or push-ups (females) performed in one minute. The physical battery immediately followed the neurocognitive battery.

### ***Biochemical Assays***

Measurement of serum cortisol, catecholamines, total protein and lactate was performed before and after each session. In addition, blood was drawn for PB, DEET, and permethrin assays at 0800, 1000, 1200, 1600, and 2400 on day one of each exposure session, as well as 0800 (immediately prior to the stress or rest period), 0900 (immediately after stress or rest), 1000, and 1200, of day two. Our highly sensitive gas chromatography/mass spectrometry (GC-MS) method for simultaneous determination of DEET and permethrin, and separate high-pressure liquid chromatography (HPLC) method for PB determination, have been reported.<sup>28</sup>

### ***Sample size calculations***

Utilizing a two-tailed t-test for a single comparison between performance on the S-CAT after treatment vs. after placebo, a standardized effect size of 0.6, an  $\alpha$  of .05 and a  $\beta$  of 0.1, we derived a sample size of 58 (or 44 with a  $\beta$  of 0.2). Then, to confirm whether the projected sample size would have sufficient power to detecting a significant difference in the proposed study, the data from another study that included the S-CAT was evaluated for the effect size associated with changes observed over three consecutive baseline trials. The evaluation of effect size on the basis of change across non-treatment baseline trials represents the most conservative

estimate for a power analysis. A conservative estimate was used to determine if the proposed sample size would result in sufficient power to detect even a very small significant change as a result of the experimental conditions. The analysis of repeated baseline S-CAT trials produced an effect size of .07. A single factor, four level (univariate one-way) repeated measures analysis for power was conducted using an effect size=.07,  $n=64$ , and  $\alpha=.01$ . The alpha value of .01 is based on a Bonferroni correction of  $\alpha=.05$  adjusted for the five SCAT tests in the battery, each of which produces an efficiency outcome score. When the effect size obtained from the analysis of change over baseline trials is combined with the projected sample size and significance level, the power to detect a very small significant change with those given parameters can be determined. This analysis yields a power of .85 for the ability to detect a significant change from the control condition with the proposed sample size. 64 participants were targeted to facilitate even block randomization.

#### *Statistical Analysis*

SAS (Version 8, SAS Institute Inc., Cary, NC) was used for statistical data management and data analysis. To compare the difference between the four exposure groups, a generalized linear model (SAS GENMOD procedure) was used to analyze repeated measures (e.g., blood pressure and heart rate); general linear model (SAS GLM procedure) or student's t-test was used to compare mean physical performance measures or change in blood pressure from baseline to peak. The Wilcoxon rank test was used to assess whether baseline characteristics were associated with outcome measures. Statistical results were considered significant if the p value was less than 0.05 (2-tailed). Analyses were performed using data for all 81 participants, except for WinSCAT analyses, which require full data for all sessions, available for 63 of 64 completers. Another 12 individuals were consented, but withdrew prior to an exposure session; they are included in Figure 1 but had no outcome measures. Intent-to-treat analysis and missing value imputation were not used.



## RESULTS

Sixty-four participants completed all four exposure combinations, as targeted in the study design, while another 17 completed one to three exposure sessions, but due to deployment or schedule conflicts did not complete the full study. Table 1 provides demographic and baseline data for both the 64 completers as well as all 81 participants, in separate columns. Participants had excellent functional status, demonstrated by their SF-36 scores. Three subjects reported moderate symptoms of depression on the PHQ-9 (two with scores of 10, one with a 13), and 4 had mild depressive symptoms (scores of 5 or 6).

Permethrin was never detected in the plasma of any participant, with a detection limit of 5 ng/ml, whereas DEET was consistently detected in sessions where participants were exposed; there were no significant differences between the maximum concentration of DEET ( $C_{max}$ ), the time when the maximum concentration was reached ( $T_{max}$ ), or the area under the curve (AUC) for DEET, by participant gender, weight, or body mass index (BMI), or stress exposure. However, mean PB levels were significantly higher immediately after stress than rest periods, but this difference did not persist (Figure 2). Total protein levels increased 7.5% post-stress vs. pre-stress, indicating stress-induced hemoconcentration accounted for part of this difference. PB AUC and  $C_{max}$  were both significantly inversely correlated with body weight and BMI under stress, but not rest, conditions. No pharmacodynamic measures significantly differed by sex. No relationship could be discerned between either DEET or PB concentrations and neurocognitive or physical outcome measures.

Systolic blood pressure and heart rate increased markedly during stress sessions, without difference according to treatment status (Figure 3). Epinephrine, norepinephrine, and lactate levels likewise increased significantly with stress, regardless of treatment status. No significant changes in diastolic pressure, dopamine, or cortisol were observed with any exposure combination.

Neurocognitive performance, measured by the WinSCAT, did not differ by treatment. However, stress modestly enhanced subsequent neurocognitive performance, through improvement in several WinSCAT tasks (Table 2). Global neurocognitive performance was better after stress than rest for placebo exposures ( $p < .01$ ), with a non-significant trend favoring stress for treatment exposures ( $p = .16$ ; Figure 4). Neurocognitive outcomes did not differ by BMI or gender.

Unadjusted physical outcome measures did not differ by exposure group (Table 3). However, multiple regression analyses, incorporating participant gender and BMI, discerned that handgrip duration was significantly worse after stress than rest ( $p < 0.01$  with treatments;  $p = 0.015$  with placebos). Maximum handgrip strength was significantly greater for males than females ( $p < 0.0001$ ), which resulted in a difference in handgrip integral ( $p < 0.001$ ), despite similar handgrip duration. No other physical outcome measures differed by weight, BMI or gender.

Side effects were rare and did not differ by treatment (Table 4).

## **DISCUSSION**

Previous investigators, who exposed animals for 60 days to unusually high doses, or unusual administration methods, of PB, DEET, and permethrin, have extrapolated from their data to suggest that combined treatment may be harmful when used as indicated.<sup>12,21,23</sup> Our study, the first to rigorously examine physical and neurocognitive effects of appropriate doses and routes of administration of combined treatments in human subjects, counters such suggestions. We found no evidence that the short-term combined use of PB, DEET, and permethrin has any adverse impact on physical or neurocognitive performance in humans. Moreover, short-term stress did not facilitate treatment induced neurocognitive impairment. If anything, stress enhanced neurocognitive performance. There are several potential explanations for the difference between our findings and other studies. The most significant is the degree of exposure: many therapies

are effective when used appropriately, but harmful with excessive exposure. Permethrin exposure in animal studies is particularly problematic, since we found that permethrin-treated uniforms did not lead to measurable bloodstream permethrin. It is also important to recognize that few GWVs had permethrin-treated uniforms,<sup>17</sup> and there is no evidence that these veterans were disproportionately ill, which renders permethrin an unlikely factor in Gulf War Syndrome. Animal studies utilized permethrin by subcutaneous injection (hens) or application to preclipped skin on the posterior neck (rats). Either method represents direct, unequivocally greater exposure than with appropriate human use. Permethrin, administered appropriately, has demonstrable safety in humans, but at excessive dosages, caused neurotoxicity in rats,<sup>29</sup> that could explain the pathology described in animal studies. Rodent skin is far more permeable to permethrin than human skin,<sup>19</sup> and the duration of exposure is markedly different: 60 days' exposure in animal studies versus 24+ hours in our study. We believe our findings have greater applicability to deployed soldiers.

Pyridostigmine bromide, at the dose used, is believed to inhibit 20-40% of acetylcholinesterase at the neuromuscular junction.<sup>1</sup> Whereas there is enough AChE to maintain normal function under most conditions, we expected that maximal physical stress might induce measurable differences in performance, especially in handgrip strength and duration. We also anticipated occasional muscarinic or nicotinic effects of excess acetylcholine, such as fasciculations, heart rate variability, and bladder or bowel symptoms. No symptoms were more common with treatment than placebo, which elucidates the importance of assessing "side effects" scientifically, rather than by retrospective self-report. Surprisingly, no evidence for a differential effect of PB on performance by weight, BMI, or gender was noted, which indicates the same prophylactic dose is appropriate for all young adults. We conclude that short-term use of the 30 mg thrice-daily PB dose is safe and well tolerated. We can not, however, comment

upon efficacy, which must be based on animal studies or observations after terrorist or wartime exposure, since it is unethical to deliberately expose humans to nerve agents.

That physical and mental stress increased PB levels is novel, and attributable to at least two factors. First, stress-induced diaphoresis depleted plasma volume, as evidenced by the 7.5% increase in total protein levels; second, strenuous exertion may have diverted blood flow away from the kidneys and toward skeletal muscles to reduce PB excretion. The most salient finding is that even when stress induced significantly higher PB levels, there was no impairment of physical or neurocognitive function.

The most significant limitation of our study is its short-term nature. Our results cannot necessarily be extrapolated to conclude that long-term exposure is safe, nor that long-term stress would have similar effects, but it is unlikely that troops, or civilians threatened by terrorism, would take PB for a prolonged duration, so our study closely approximates realistic doses, routes of administration, *and* durations. However, our combined stressors do not approximate the real stress of combat or terrorism, and while short-term stress seemed to “prime” subjects, enhancing neurocognitive performance, chronic stress might impair performance. Nevertheless, it is unlikely that appropriately used PB or insect repellents would exacerbate impairment. Since some Gulf War veterans may have used repellents or PB in inappropriate ways or doses, with potential adverse effects, so our results should not be construed to conclude that such exposures did not impair any Gulf War veterans. Another limitation of our study is the healthy population, facilitating the application of our results to a deployed military population, but limiting generalization to a more diverse civilian population faced with terrorism. Consideration should be given to study of a more diverse population.

Future research might include chronic exposure to study treatments and chronic stress. Given no significance for permethrin-treated uniforms, it may be worthwhile to repeat chronic exposure animal studies utilizing only PB and DEET. Since insect repellents are often used in

tropical environments, it may also be worthwhile to repeat our study under conditions of higher humidity and warmer temperatures.

### CONCLUSIONS

Short-term exposure to combined treatment with PB, DEET, and permethrin was remarkably well tolerated and did not impair physical or neurocognitive performance in our study population. Individuals threatened by disease-carrying insects and/or nerve agents should have confidence that these preventive measures are not harmful.

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**TABLE 1. Subject Demographics and Baseline Data (n = 81)**

Sex	n = 81 participants	n = 64 completers
Male	54 (67%)	41 (64%)
Female	27 (33%)	23 (36%)
Race		
Native American	1 (1%)	0
African-American	6 (9%)	4 (6%)
Latino	10 (15%)	6 (9%)
Asian	5 (6%)	7
Caucasian	58 (72%)	47
Age (mean, years)	28.1	28.4
(range)	21-49	21-49
BMI (mean)	25.6	
PHQ-9 score (median)	0	0
SF-36 subscale scores (median)		
General Health	90.0	
Mental Health	88.0	
Physical Functioning	100.0	
Social Functioning	100.0	
Emotional	100.0	
Bodily Pain	84.0	
Role Physical	100.0	
Vitality	70.0	
PCL-C (median)	19	

**Table 2: Neurocognitive Outcomes (WinSCAT Results by Task; n=63 completers with full data)**

<u>Task</u>	<u>Stress/Rx</u>	<u>Rest/Rx</u>	<u>Stress/Pl</u>	<u>Rest/Pl</u>
Code Memory	60	60	61	58*
Code Match	66	64#	67	65*
Running Memory	133	131	133	130
Matching	44	44	42	42
Math	34	33	34	31^

\*different than stress/placebo combination,  $p < 0.05$

#different than stress/treatment combination,  $p < 0.05$

^different than both stress/treatment and stress/placebo combinations,  $p < 0.05$

**Table 3: Physical Outcomes (n=81 completing at least one exposure session)**

<u>Task</u>	<u>Exposure</u>			
	Stress/Rx	Rest/Rx	Stress/Placebo	Rest/Placebo
Max Handgrip	83.3	84.5	85.5	82.3
Strength (mmHg)				
Handgrip Duration	187	209	191	214
(mmHg)				
Handgrip Integral	4283	4664	4400	4957
Harvard Steps	47.2	47.0	47.7	47.3
Push-ups	37.8	37.3	37.9	38.1
(Females Only)				
Pull-ups	6.5	6.0	6.5	6.1
(Males Only)				

**Table 4: Side Effects (Number, by Self-Report, for 81 subjects completing at least 1 session)**

<u>Symptom</u>	<u>Exposure</u>			
	Stress/Rx	Rest/Rx	Stress/Placebo	Rest/Placebo
Fatigue	2	1	4	1
Pruritis/Rash	2	2	2	2
Headache	3	1	2	2
Gastrointestinal	3	3	4	4
Viral URI	3	2	4	2
Muscle Twitch	1	1	2	1
Diaphoresis	1	1	2	0
Tachycardia	0	2	2	0
Bradycardia	1	2	0	1
Memory Problems	0	1	2	1
Nightmares	0	2	2	0

## **Figure Legends**

**Figure 1:** Study flow diagram

**Figure 2:** Pyridostigmine Bromide levels for stress vs. rest sessions

**Figure 3:** Hemodynamic Changes (Maximum increase during session compared to baseline)

**Figure 4:** Overall Neurocognitive Performance (WinSCAT)

Figure 1

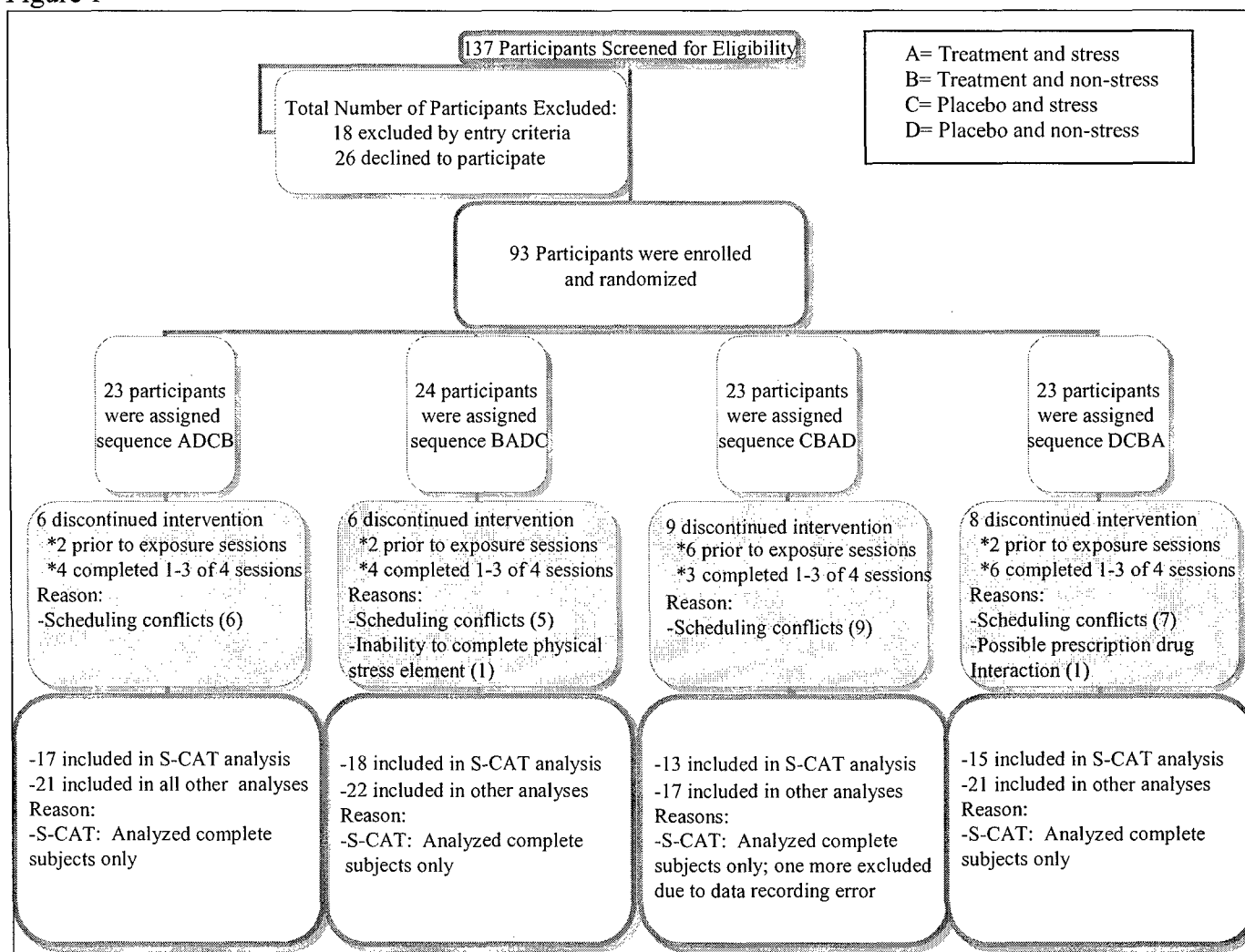




Figure 2

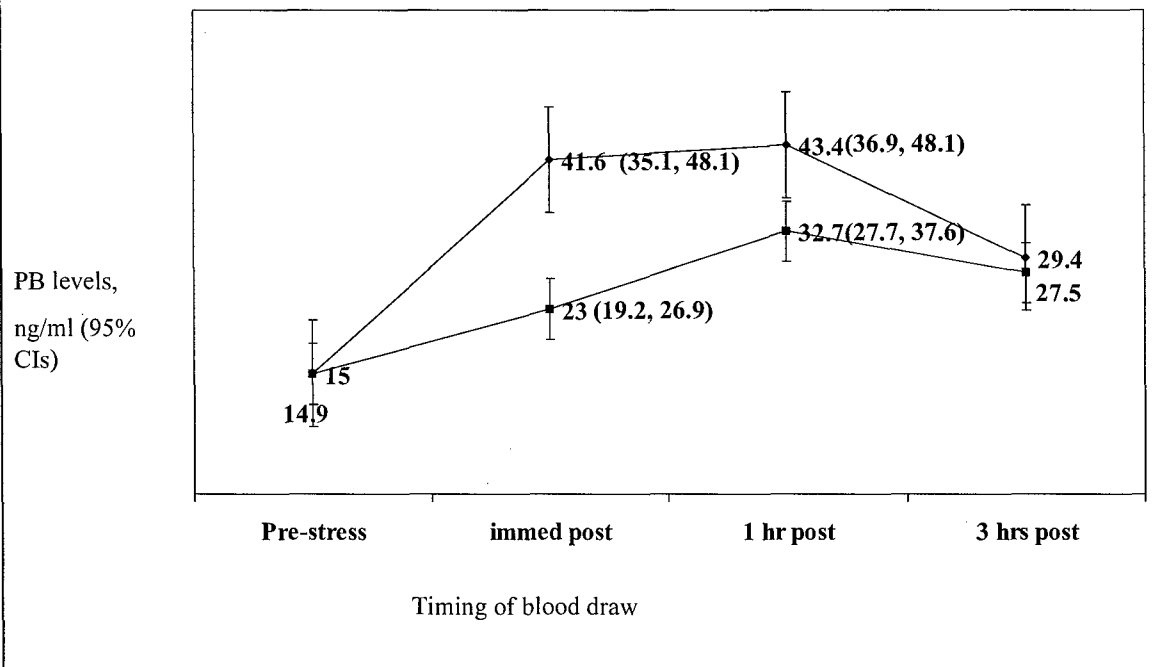


Figure 3

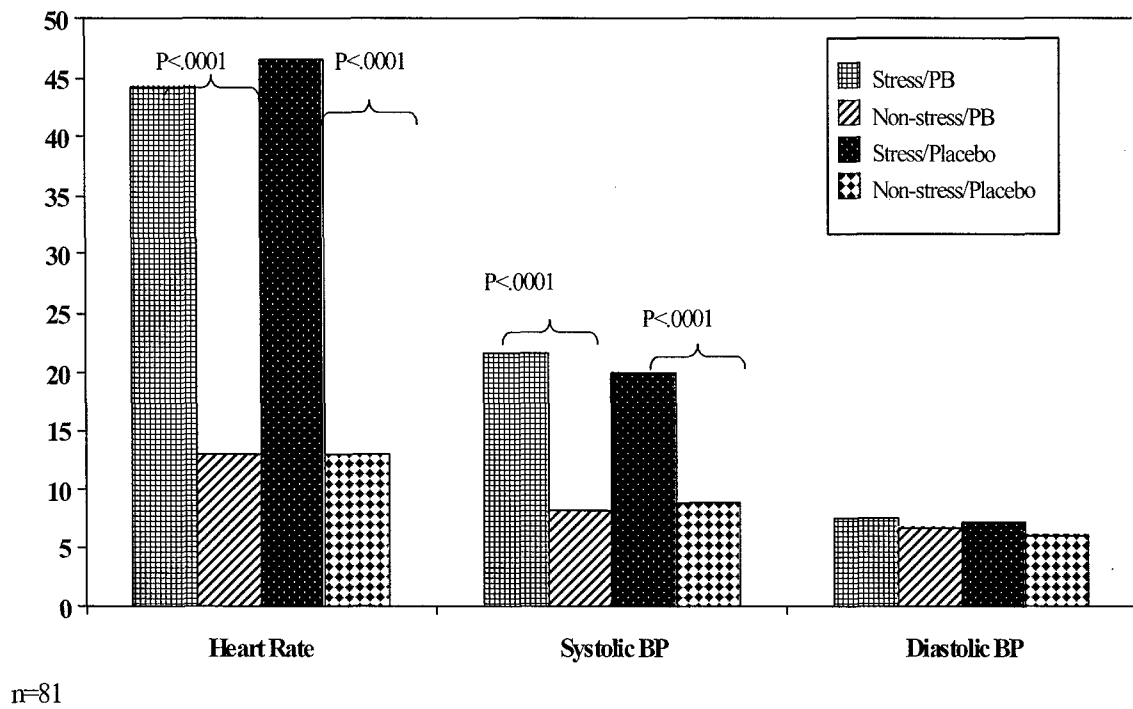
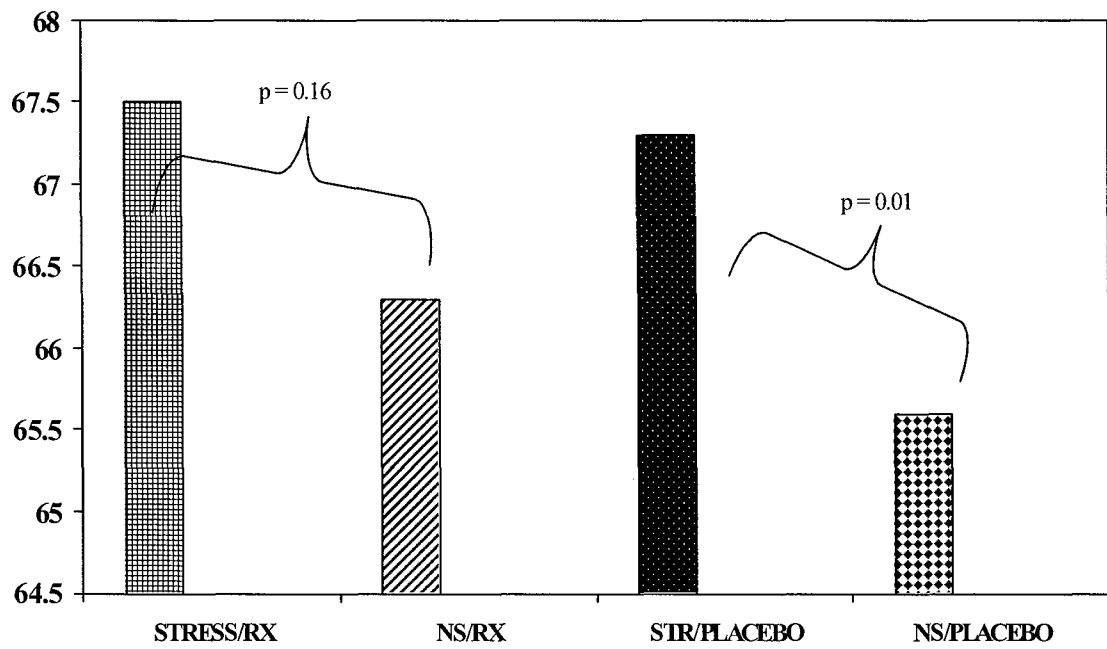


Figure 4



n=54