AD_____

AWARD NUMBER: W81XWH-16-2-0050

TITLE: The Role of “Desert-Dust” Metals in the Pathobiology of Gulf War Illness

PRINCIPAL INVESTIGATOR: John F. Kalinich, PhD

RECIPIENT: Henry M. Jackson Foundation for the

Advancement of Military Medicine
Bethesda, MD 20817

REPORT DATE: October 2019

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE
October 2019

2. REPORT TYPE
Annual

3. DATES COVERED
30 Sept 18 – 29 Sept 19

4. TITLE AND SUBTITLE
The Role of "Desert-Dust" Metals in the Pathobiology of Gulf War Illness

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-16-2-0050

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
John F. Kalinich, PhD

E-Mail: john.kalinich@usuhs.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Henry M. Jackson Foundation for the
Advancement of Military
Medicine
6720A Rockledge Drive
Bethesda, MD 20817

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

After the First Persian Gulf War (1990-1991), many U.S. personnel reported suffering from a chronic multi-symptom disease eventually called "Gulf War Illness". We hypothesize that exposures to pyridostigmine bromide, permethrin, and/or DEET adversely affect the permeability of the blood-brain barrier allowing metals solubilized from inhaled desert dust particles to enter the brain. As a consequence, normal metal homeostasis is disrupted resulting in extensive oxidative damage and neurological dysfunction. In Year 3, based upon trans-endothelial electrical resistance (TEER) readings, we have successfully established an in vitro blood-brain barrier model. In some but not all cases, treatment with a variety of Gulf War-associated chemicals affected TEER values. In addition, with some treatments, changes in expression of gap junction proteins, ZO-1 and occludin were also observed.

15. SUBJECT TERMS
Gulf War Illness, desert dust, metals, DEET, permethrin, pyridostigmine bromide

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	23	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

TABLE OF CONTENTS

1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

In 1990-1991, the United States and its Coalition allies responded militarily to the Iraqi invasion of Kuwait. Close to 700,000 U.S. military personnel served in the resulting Persian Gulf War. Soon afterward, many of these individuals reported suffering from a chronic multi-symptom disease that was given the moniker "Gulf War Illness." For the past 25 years investigators have searched for a cause for these ailments, but as yet no definitive cause has been identified. The hypothesis of this research is that combined exposures to pyridostigmine bromide (PB), permethrin (PM), and/or DEET adversely affect the permeability of the blood-brain barrier (BBB) allowing metals solubilized from inhaled desert dust particles to enter the brain. As a consequence, normal metal homeostasis is disrupted resulting in extensive oxidative damage and neurological dysfunction. This project uses commercially available human brain microvascular endothelial cells and astrocytes in an *in vitro* blood-brain barrier model system to assess the effects of PB, PM, DEET, and their metabolites on BBB permeability. In addition, those compound(s) that affect BBB permeability will be further tested for their ability to enhance the translocation of metals across the BBB.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Gulf War Illness, desert dust, metals, DEET, permethrin, pyridostigmine bromide

3. **ACCOMPLISHMENTS:** The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Listed below are the five major task area associated with this project, start and end dates, and percentage of completion.

Major Task 1 – Experimental Preparation, Year 1/Month 1 to Year 1/Month 5, 100% completed.

Major Task 2 – Assessment of Cell Viability after Test Compound Exposures, Year 1/Month 6 to Year 1/Month 10, 100% completed.

Major Task 3 – Determination of BBB Permeability Changes after Administration of Test Compounds, Year 1/Month 11 to Year 2/Month 5, 90% completed.

Major Task 4 – Determination of Indicators of Oxidative Stress and Inflammation in BBB Cells after Exposure to Test Compounds, Year 2/Month 6 to Year 3/Month 2, 45% completed.

Major Task 5 – Data Compilation, Statistical Analysis, and Preparation of Final, Year 3/Month 3 to Year 3/Month 12, 20% completed.

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

In Year 3, the majority of the research effort was dedicated to solving the myriad of issues encountered with attempting to reconstitute a functional blood-brain barrier. These impediments were finally resolved. Using the transwell insert system, human astrocytes obtained from ScienCell Research were plated on the basolateral side of the transwell insert. After allowing 4 h of incubation for cell attachment, the transwell was inverted and brain microvascular endothelial cells purchased from AngioProteomie were plated on the apical side of the insert for 24 h. Commercially-available cells from numerous vendors were tested in this system, but it was found that the combination of the ScienCell Research astrocytes and AngioProteomie endothelial cells resulted in the best intact model of a functioning blood-brain barrier as assessed by trans-endothelial electrical resistance (TEER) readings (Appendices, Figure 1).

Treatment of the blood-brain barrier model system with sub-toxic concentrations (1 µM) of Gulf War-associated compounds significantly affected TEER values in some cases (e.g., pyridostigmine bromide, DEET, permethrin), but not in others (e.g., N,N-diethyl-*m*-hydroxymethylbenzamide [a DEET metabolite], 3-Phenoxybenzoic acid [a permethrin metabolite]) (Appendices, Figures 2-4).

Treatment of the blood-brain barrier model system with pyridostigmine bromide, DEET, or permethrin (1 µM) followed by treatment with nickel (1 µM) did not affect translocation of the nickel across the blood-brain barrier as compared to untreated controls (Appendices, Table 1). Combinations with other metals are currently being assessed.

Proper functioning of the blood-brain barrier depends in part on the establishment of tight junctions between the cells comprising the barrier. Two proteins involved in this are occludin, an integral membrane protein located at tight junctions, and zonula occludens-1 (ZO-1), a peripheral membrane protein responsible for the assembly of tight junctions. Using the Protein Simple Wes System we have done a preliminary assessment of the effect of treatment with Gulf War-associated compounds and their metabolites on expression of ZO-1 and occludin in human brain microvascular endothelial cells. As seen in Figure 5 of the Appendices, several compounds, pyridostigmine bromide in particular, affect expression of these critical proteins. Work continues on this finding.

Because of the issues encountered with establishing the reconstituted blood-brain barrier model system, a one-year no-cost extension of the project was requested and approved. In the final year of this project we will complete the assessment of oxidative stress and inflammatory markers induced by exposure to Gulf War-associated compounds and quantitate metal translocation across the blood-brain barrier.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Nothing to report.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to report.

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

In the next reporting period we will complete assessment of oxidative stress and inflammatory markers induced after administration of test compounds, quantitate metal translocation across the blood brain barrier, and prepare and submit the final report.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal

disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Nothing to report.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to report.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to report.

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to report.

5. CHANGES/PROBLEMS: The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project

or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

There is no change in the project objectives or scope of the research.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

After solving the issues encountered with developing a functional blood-brain barrier model system in Year 3, we anticipate no further problems with the project.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to report.

Significant changes in use or care of vertebrate animals

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS: List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

· **Publications, conference papers, and presentations**
Report only the major publication(s) resulting from the work under this award.

Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report.

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report.

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Nothing to report.

· **Website(s) or other Internet site(s)**
List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report.

· **Technologies or techniques**
Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to report.

· **Inventions, patent applications, and/or licenses**
Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to report.

· **Other Products**
Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

- *data or databases;*
- *physical collections;*
- *audio or video products;*
- *software;*
- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?
Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Example:

Name: *Mary Smith*
Project Role: *Graduate Student*
Researcher Identifier (e.g. ORCID ID): *1234567*
Nearest person month worked: *5*

Contribution to Project: *Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support: *The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

Name: John Kalinich, PhD
Project Role: Team Leader
Researcher Identifier: 0000-0003-1591-9389
Nearest person month worked: 2
Contribution to Project: Responsible for overall functioning of this portion of the project.
Funding Support: Federal Government Employee (Department of Defense)

Name: Christine Kasper, PhD RN, FAAN FACS
Project Role: Co-investigator,
Research Identifier: 0000-0002-7784-2519
Nearest person month worked: 1
Contribution to Project: Responsible for experimental planning
Funding Support: Federal Government Employee (Department of Veterans Affairs)
Note: Dr. Kasper has moved to the University of New Mexico

Name: Jessica Hoffman, PhD
Project Role: Co-investigator
Researcher Identifier: 0000-0003-1858-8394
Nearest person month worked: 5
Contribution to Project: Responsible for establishment of cell model systems and testing.
Funding Support: Federal Government Employee (Department of Defense)

Name: Anya Fan, MS
Project Role: Research Assistant
Nearest person month worked: 12
Contribution to Project: Responsible for cell culture maintenance and determination of oxidative stress markers.
Note: Ms. Fan joined the project in August 2019.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to report.

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

Not Applicable.

QUAD CHARTS: If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.

Not required.

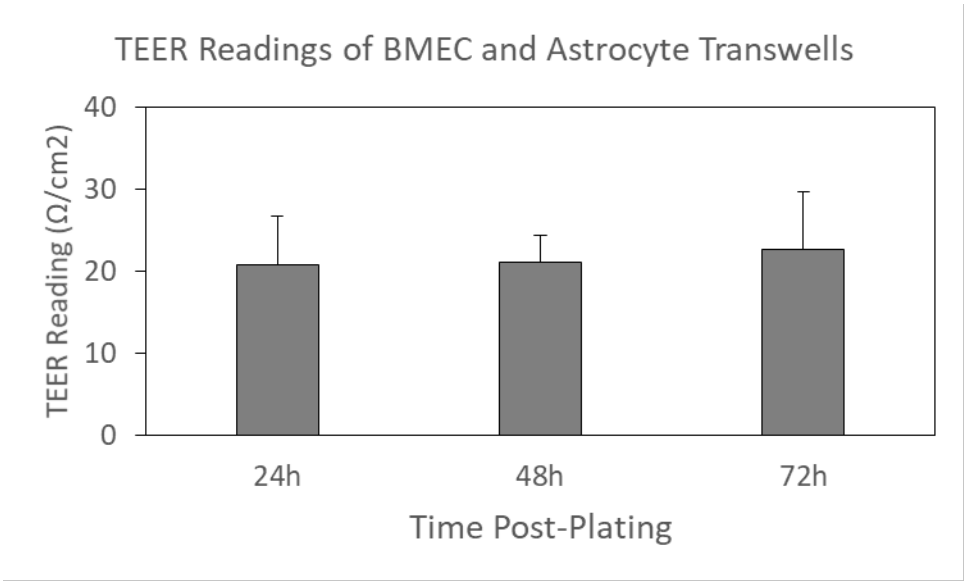
9. APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

APPENDICES

Abbreviations Used

Al	Aluminum
BBB	Blood Brain Barrier
BMEC	Brain Microvascular Endothelial Cells
Co	Cobalt
Cu	Copper
DEET	N,N-Diethyl-*m*-toluamide
DEET-ET	N-ethyl-*m*-toluamide
DEET-OH	N,N-diethyl-*m*-hydroxymethylbenzamide
Di-PB	3-Hydroxy-1-methylpyridinium bromide
DMSO	Dimethylsulfoxide
DU	Depleted uranium
Fe	Iron
LDH	Lactate dehydrogenase
Mn	Manganese
Ni	Nickel
NR	Neutral Red
PB	Pyridostigmine bromide
PB-OH	3-Hydroxy-1-methylpyridinium bromide
PM	Permethrin
PM-acid	3-Phenoxybenzoic acid
PM-alcohol	3-Phenoxybenzyl alcohol
Sr	Strontium
TEER	Trans-endothelial Electrical Resistance
Zn	Zinc

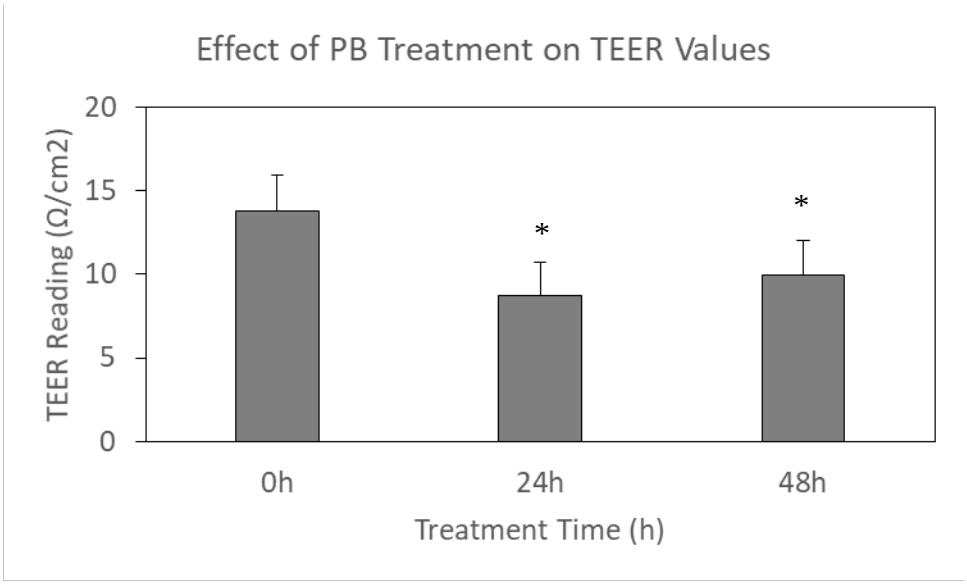
Figure 1: Trans-Endothelial Electrical Resistance (TEER) Values of Transwell Human Brain Microvascular Endothelial Cells (Apical Side) and Human Astrocytes (Basolateral Side) Over Time.



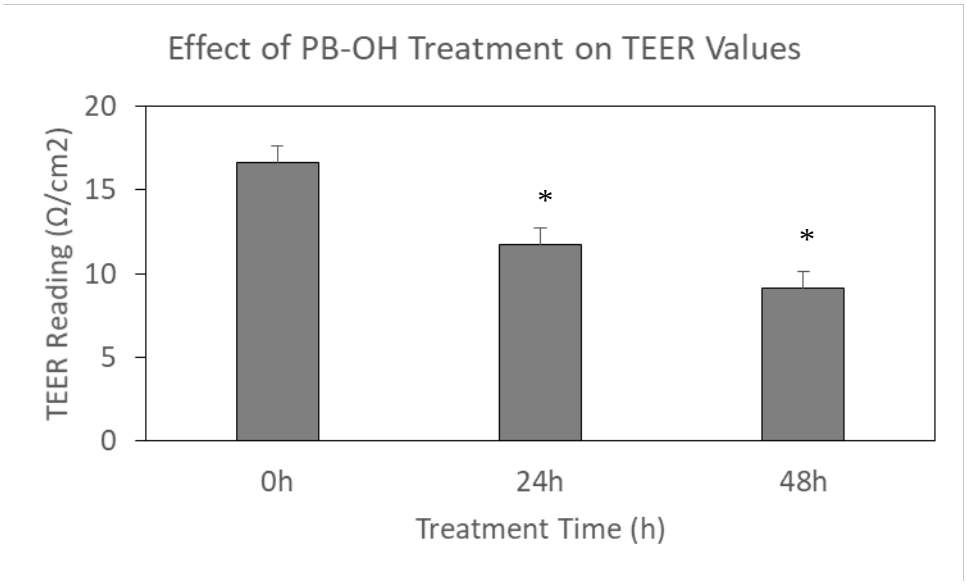
Human astrocytes (ScienCell Research) were plated on the basolateral side of a transwell insert and incubated for 4h at 37ºC to allow for attachment. After that time, the transwell was inverted and human brain microvascular endothelial cells (AngioProteomie) were added to the apical side and the plate returned to the incubator for 24h. TEER readings were taken with a EVOM2 Voltohmmeter (World Precision Instruments) using STX2 Electrodes at 24, 48, and 72h post-plating. Readings of control transwells (no cells, medium only) were subtracted from the experimental readings and resistance calculated (Ω/cm^2). Data represent the mean of six independent determinations. Error bars denote standard deviation.

Figure 2: Effect of PB and PB-OH on Transwell TEER Values

A



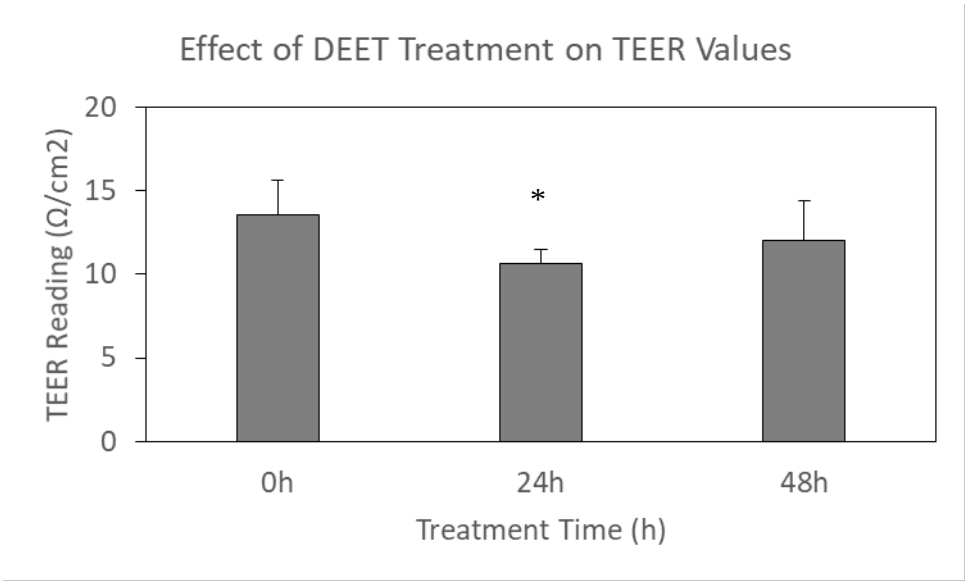
B



Transwell inserts were prepared with human astrocytes on the basolateral side and human brain microvascular endothelial cells on the apical side. Inserts were treated with 1 µM of pyridostigmine bromide (PB) or 3-hydroxy-1-methylpyridinium bromide (PB-OH). TEER values were obtained prior to treatment (0h) and at 24h and 48h post-treatment. Data represent the mean of 6 determinations. Error bars are standard deviation. Significance was assessed with a one-way ANOVA with significance set at $P<0.05$. An "*" represents a result statistically different from 0h.

Figure 3: Effect of DEET, DEET-OH, and DEET-ET on Transwell TEER Values

A



B

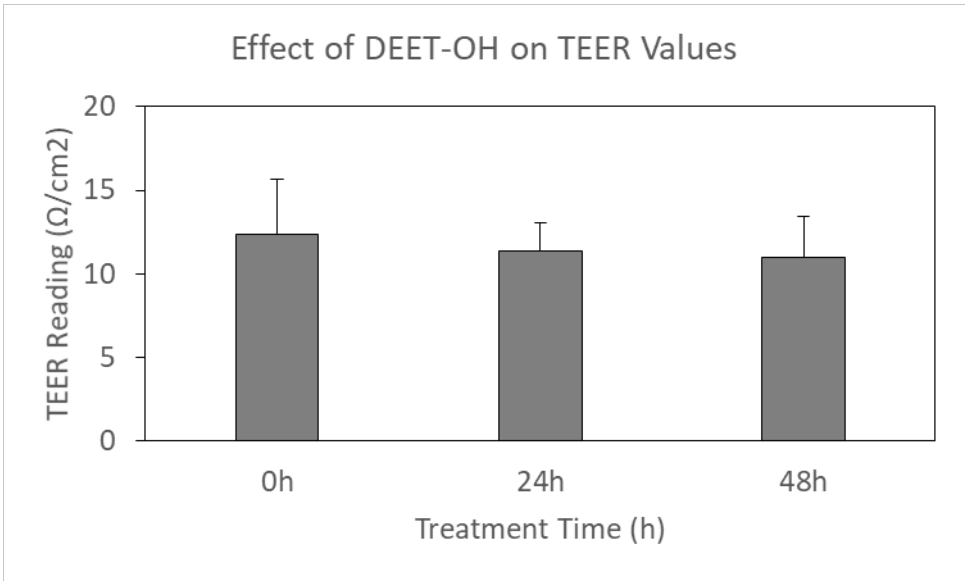
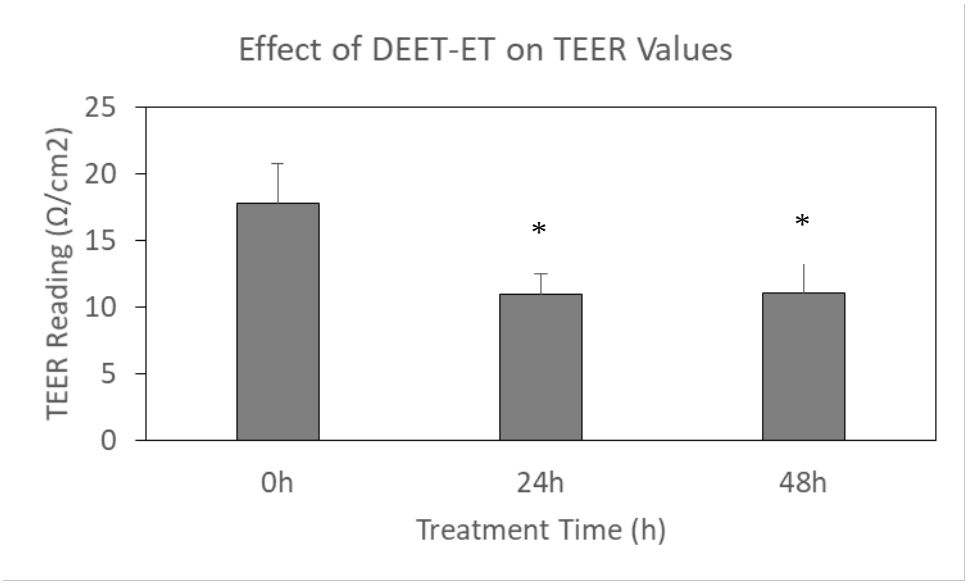


Figure 3 (cont.): Effect of DEET, DEET-OH, and DEET-ET on Transwell TEER Values

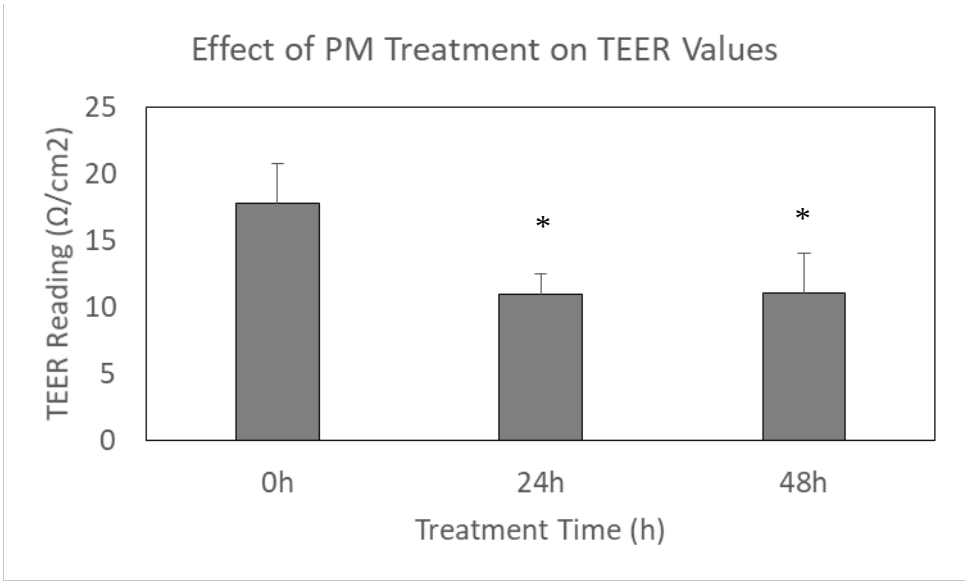
C



Transwell inserts were prepared with human astrocytes on the basolateral side and human brain microvascular endothelial cells on the apical side. Inserts were treated with 1 µM of N,N-Diethyl-*m*-toluamide (DEET), N,N-diethyl-*m*-hydroxymethylbenzamide (DEET-OH), or N-ethyl-*m*-toluamide (DEET-ET). TEER values were obtained prior to treatment (0h) and at 24h and 48h post-treatment. Data represent the mean of 6 determinations. Error bars are standard deviation. Significance was assessed with a one-way ANOVA with significance set at $P<0.05$. An "*" represents a result statistically different from 0h.

Figure 4: Effect of PM, PM-acid, and PM-alcohol on Transwell TEER Values

A



B

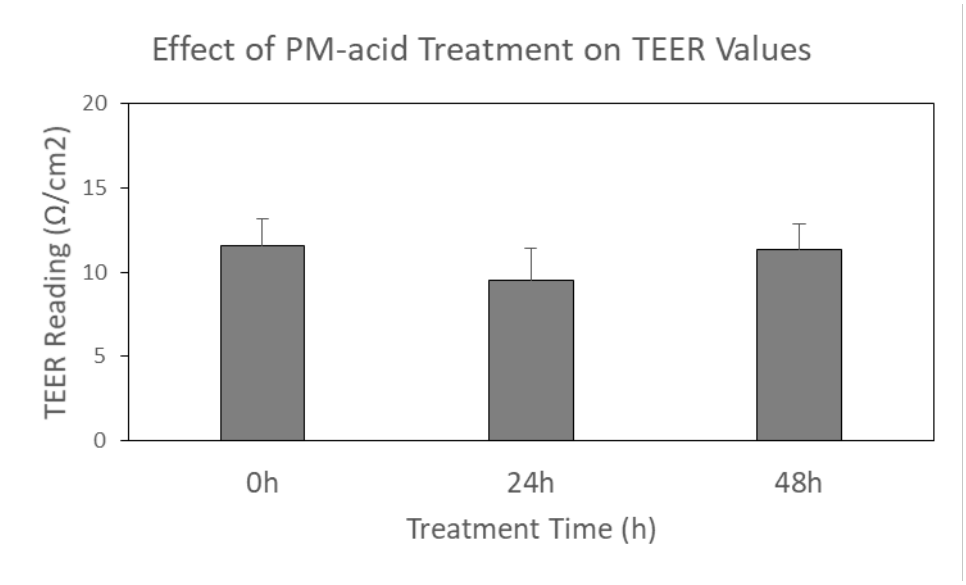
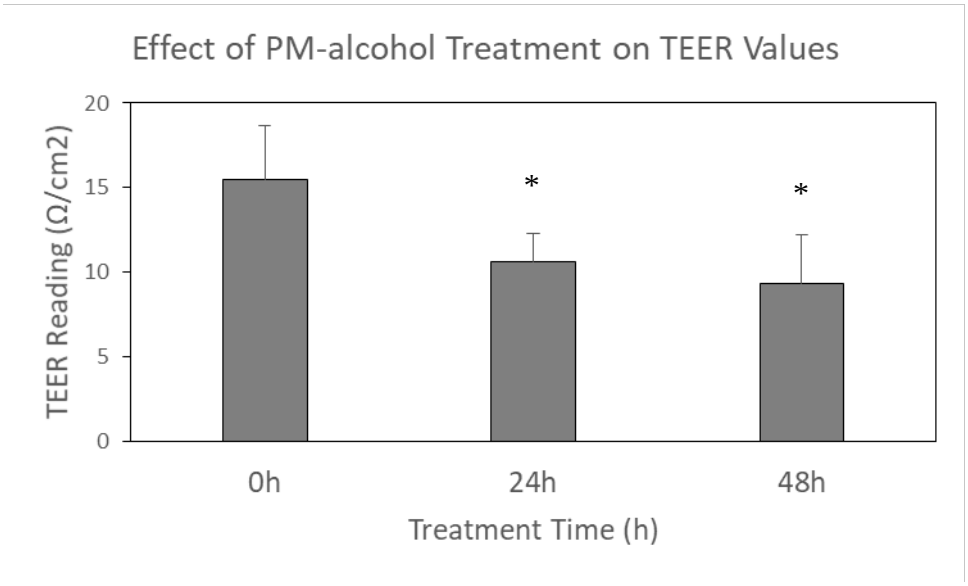


Figure 4 (cont.): Effect of PM, PM-acid, and PM-alcohol on Transwell TEER Values

C



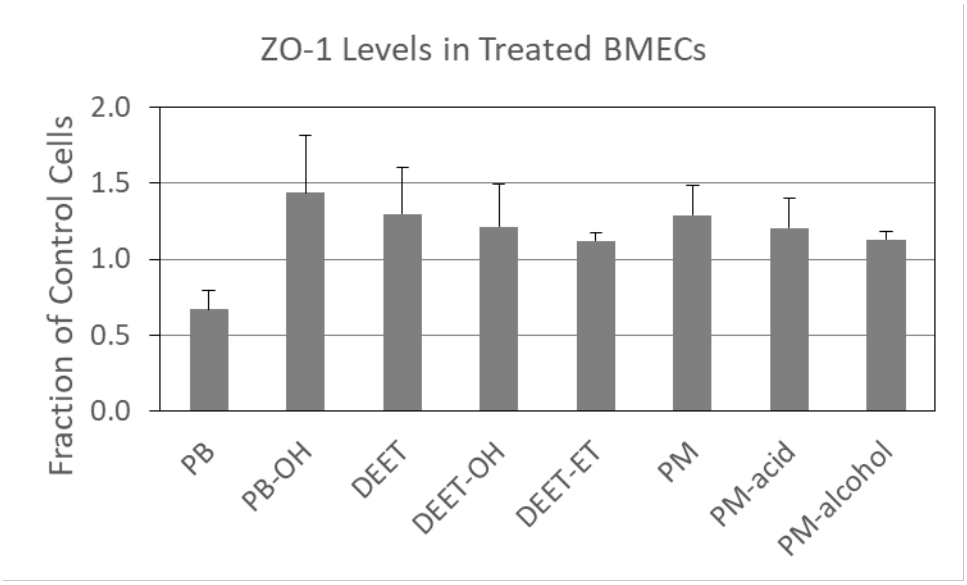
Transwell inserts were prepared with human astrocytes on the basolateral side and human brain microvascular endothelial cells on the apical side. Inserts were treated with 1 µM of Permethrin (PM), 3-Phenoxybenzoic acid (PM-acid), or 3-Phenoxybenzyl alcohol (PM-alcohol). TEER values were obtained prior to treatment (0h) and at 24h and 48h post-treatment. Data represent the mean of 6 determinations. Error bars are standard deviation. Significance was assessed with a one-way ANOVA with significance set at $P<0.05$. An "*" represents a result statistically different from 0h.

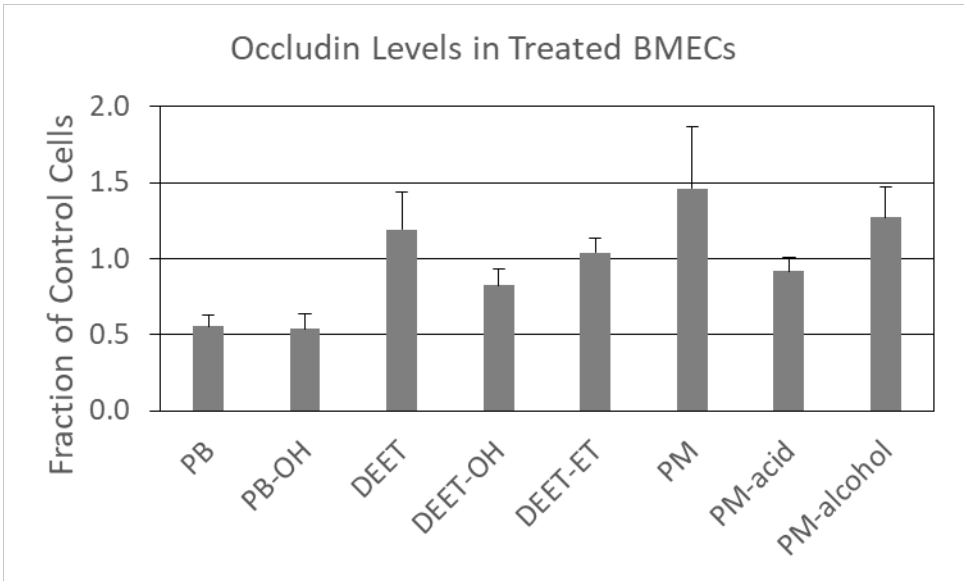
Table 1: Effect of Permethrin, DEET, or Pyridostigmine Bromide Treatment on Translocation of Nickel Across a Reconstituted Blood-Brain Barrier

Side Sampled	Control	Permethrin
BMEC	49.3%	51.7%
Astrocyte	50.7%	48.3%
	Control	DEET
BMEC	61.2%	58.0%
Astrocyte	38.8%	42.0%
	Control	Pyridostigmine
BMEC	53.1%	53.7%
Astrocyte	46.9%	46.3%

Transwell BBB models were treated with permethrin, DEET, or pyridostigmine bromide at a final concentration of 1 µM for 24h. After this time nickel chloride (1 µM Ni final concentration) was added and the system incubated for an additional 24h. Aliquots from both the apical (BMEC) side and the basolateral (astrocyte) side were taken and analyzed for nickel content using inductively coupled plasma-mass spectrometry (ICP-MS). Data are expressed as percentage of added Ni retained for each side and are the mean of six independent observations. There is no statistical difference between control and treated cells with respect to Ni translocation.

Figure 5: Protein Expression in Treated Brain Microvascular Endothelial Cells





Endothelial cells collected from the transwell membranes were mechanically homogenized with 0.5 mm glass beads and the Bullet Blender (Next Advance) and proteins detected by chemiluminescence using the ProteinSimple Wes system (ProteinSimple). Target antibodies ZO-1 (1:50, ThermoFisher) and occludin (1:50, ThermoFisher) were both normalized against b-actin (1:10,000, CellSignaling). Data are normalized to fraction of control cell expression and are presented as mean of three separate experiments with error bars representing standard deviation.