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

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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 hypothesized that exposures to pyridostigmine bromide, permethrin, and/or DEET adversely affected 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 could be disrupted resulting in extensive oxidative damage and neurological dysfunction. In this project an in-vitro blood-brain barrier model co-exposed to DEET, permethrin, or pyridostigmine bromide with desert dust-associated metals was assessed for structural and functional integrity, as well as damage markers. While most treatments had minimal effects, the combination of DEET, permethrin, or pyridostigmine bromide with depleted uranium increased nitric oxide production. In addition, various treatments also affected cytokine production in this model.					
15. SUBJECT TERMS Gulf War Illness, desert dust, metals, DEET, permethrin, pyridostigmine bromide					
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1. INTRODUCTION:

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 used 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, and DEET 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:

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

3. ACCOMPLISHMENTS:

- **What were the major goals of the project?**

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, 100% 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 4/Month 2, 100% completed.

Major Task 5 – Data Compilation, Statistical Analysis, and Preparation of Final, Year 4/Month 3 to Year 4/Month 12, 100% completed.

- **What was accomplished under these goals?**

In the first year of this project, financial accounts were established, personnel hired and trained, and all required regulatory approvals were obtained. There were issues in Year 1 obtaining the required cell lines due to a contamination event at one of the vendors. However, after numerous delays, we were finally able to secure the human astrocyte and endothelial cells required for the project. The cell lines were successfully established in our laboratory and toxicity assessments of pyridostigmine bromide, permethrin, and DEET completed. The toxicity assessments included the following tests for cell viability and function: MTT conversion, Neutral Red uptake, and lactate dehydrogenase release. In addition, changes in morphology were determined microscopically.

The toxicity of the metals to be tested in this project was also determined using the aforementioned tests in Year 1 of the project. Metals tested were those found in elevated levels in the desert dust of Iraq and Kuwait and included: aluminum, iron, uranium, nickel, cobalt, copper, strontium, manganese, and zinc. The viability assay procedures and experimental results can be found in our *International Journal of Environmental Research and Public Health* (IJERPH) manuscript in the Appendices. From these studies, non-toxic concentrations of all test components were selected for further study. These results can also be found in the IJERPH manuscript in the Appendices, but generally were in the range of 1-10 μ M for the organic compounds and 1 μ M for the metals. In Year 1 we also successfully validated the analytical methods for our test metals using inductively coupled plasma-mass spectrometry (ICP-MS). Also in Year 1, the Dynamic In Vitro Blood Brain Barrier (DIV-BBB) System from FloCel was purchased. We spent a great deal of time trying to establish the system and determine its functional characteristics. We found that even after numerous conversations with FloCel technical support we were unable to get the system to perform as advertised by FloCel. As a result, a decision was made to switch to our back-up plan of using multi-cultured transwell inserts to determine BBB permeability after test compound exposures. However, work in this area was slowed due to an inexplicable delay in obtaining the astrocyte culture medium from the original selected vendor. After numerous promised delivery dates were missed, we switched to a secondary vendor. This switch necessitated retesting our astrocyte cultures to determine that their functionality is comparable to that seen in the previously used medium.

In Year 2 of the project, work focused on developing the co-culture transwell insert model for our studies. Work was initially slowed due to issues with maintaining a confluent monolayer with tight junctions. We had extensive conversations and collaborative efforts with the NIH National Center for Advancing Translational Sciences (NCATS) and 4-D Bio-3, a three-dimensional bioprinting start-up in conjunction with the Uniformed Services University, the Naval Research Laboratories, and the Walter Reed National Military Medical Center. As shown in the IJERPH manuscript in the Appendices we were able to grow human brain microvascular endothelial cells in transwell inserts that expressed the appropriate endothelial cell-associated proteins. Our preliminary work was presented as a poster at the Society for Neuroscience meeting. The meeting abstract can be found in the Appendices. Also in Year 2, the various assays to assess oxidative stress and inflammatory biomarkers were standardized.

In Year 3, the effects of DEET, permethrin, and pyridostigmine bromide, as well as desert dust metals, alone and in combination, on a variety of oxidative stress biomarkers were assessed. Markers analyzed included nitric oxide production, protein carbonyl damage, and formation of thiobarbituric acid reactive substances (an indication of lipid peroxidation damage). The results of these studies showed no overt damage after exposure to either the organic compounds or metals, alone or with co-exposure, with the exception of a significant increase in nitric oxide production after treatment with the three organic compounds tested in combination with depleted uranium. These results can be found in the IJERPH manuscript located in the Appendices. Also in Year 3, the effect of organic and/or metal exposure on a series of oxidative stress proteins was assessed using the Protein Simple WES System. The following proteins from the treated brain microvascular endothelial cells were analyzed: glutathione peroxidase-4; catalase; superoxide dismutase; matrix metalloproteinase-3; and matrix metalloproteinase-9. No statistically significant differences were found. The results of these experiments can be found in the IJERPH manuscript

in the Appendices. Because not all of the proposed work had been completed we requested and were granted a one-year no-cost extension for the project.

In Year 4, assessments of blood-brain barrier structure and function after organic compound and/or metal exposure were completed. Two tests of BBB function were used: trans-endothelial electrical resistance (TEER) and fluorescent dextran translocation. TEER is used to determine the integrity of the endothelial cell monolayer on the transwell insert. The translocation of fluorescent dextran used two dextran compounds of different molecular weights (e.g., 4 kDa and 70 kDa) to assess the ability of the artificial BBB to maintain its barrier function in the presence of the test compounds. The results of these studies are found in our manuscript submitted for publication in *Life Sciences*. Both the data and manuscript submission are found in the Appendices. In addition, the effect of organic compound plus/minus metals on proteins involved in tight junction formation of the BBB was also assessed. The expression of the following proteins were analyzed using the Protein Simple WES System: occludin, ZO-1, and claudin-5. Again, results can be found in the *Life Sciences* manuscript in the Appendices. The ability of the various desert dust metal to translocate from the luminal side to the abluminal side of the BBB was investigated using inductively coupled-plasma mass spectrometry (ICP-MS). The data from these experiments can be found in the Appendices in the *Life Sciences* manuscript submission. Finally, the effect of organic compound plus/minus metal exposure on cytokine production was also assessed in the final year of the project. The Meso Scale Discovery U-Plex human biomarker kit was used for these determinations with results found in the *Life Sciences* manuscript submission in the Appendices. Briefly, while several of the co-exposures resulted in reduced TEER values, minimal to no effects were observed on the translocation of the fluorescent markers or metals across the BBB. In addition, expression of tight junction proteins was also not statistically different; however, several cytokines increased synergistically with several of the co-exposure scenarios. Of all the combinations, pyridostigmine bromide, permethrin, and depleted uranium had the most consistent results. Again these data can be found in the *Life Sciences* manuscript submission shown in the Appendices. Finally in Year 4, our laboratory presented some of our results at the CDMRP-sponsored virtual State-of-the-Science meeting. The abstract and slides for this meeting can also be found in the Appendices.

• **What opportunities for training and professional development has the project provided?**

Nothing to report

• **How were the results disseminated to communities of interest?**

Results from this project were disseminated to communities of interest through the publication of the collected data in peer-reviewed scientific journals and through participation in meetings with other Gulf War Illness researchers and community representatives.

• **What do you plan to do during the next reporting period to accomplish the goals?**

Nothing to report.

4. IMPACT:

• **What was the impact on the development of the principal discipline(s) of the project?**

The findings from this study indicates that there are subtle changes on the blood-brain barrier after co-exposure to Gulf War-associated organic compounds and metals found in the desert dust of the region. It also suggests that, heretofore unstudied, in-theatre inhalation exposures may play a far greater role in adverse health effects than originally thought.

- **What was the impact on other disciplines?**

Nothing to report.

- **What was the impact on technology transfer?**

Nothing to report.

- **What was the impact on society beyond science and technology?**

Nothing to report.

5. CHANGES/PROBLEMS:

- **Changes in approach and reasons for change**

Nothing to report.

- **Actual or anticipated problems or delays and actions or plans to resolve them**

Nothing to report.

- **Changes that had a significant impact on expenditures**

Nothing to report.

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

Nothing to report.

6. PRODUCTS:

Publications, conference papers, and presentations

- **Journal publications.**

Jessica F. Hoffman and John F. Kalinich. "Effects of Incubation of Human Brain Microvascular Endothelial Cells and Astrocytes with Pyridostigmine Bromide, DEET, or Permethrin in the Absence or Presence of Metal Salts". *International Journal of Environmental Research and Public Health* 17 (22): 8336 (2020) – doi: 10.3390/ijerph17228336. – acknowledgement of federal support – Yes.

Jessica F. Hoffman, Vernieda B. Vergara, Anya X. Fan, and John F. Kalinich. The Gulf War-associated compounds DEET, pyridostigmine bromide, and permethrin, in combination with desert dust metals, have subtle adverse effects on blood brain barrier function in an in-vitro model. *Life Sciences* (manuscript submitted) – acknowledgement of federal support – Yes.

- **Books or other non-periodical, one-time publications.**

Nothing to report.

- **Other publications, conference papers, and presentations.**

Jessica F. Hoffman, Christine E. Kasper, and John F. Kalinich. Effect of Gulf War “Desert-Dust” compounds on the viability and permeability of the blood-brain barrier in a 3D model. Poster Session, Society for Neuroscience Meeting, 11-15 November 2017, Washington, DC.

Jessica F. Hoffman, Anya X. Fan, Vernieda B. Vergara, and John F. Kalinich. Effects of co-exposure to organic compounds and “desert dust” metals on the integrity of the blood brain barrier: a possible cause of Gulf War Illness. Poster Presentation, Gulf War Illness State of the Science Virtual Meeting, August 2020.

- **Website(s) or other Internet site(s)**

Nothing to report.

- **Technologies or techniques**

Nothing to report.

- **Inventions, patent applications, and/or licenses**

Nothing to report

- **Other Products**

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Name: John Kalinich, PhD

Project Role: Principal Investigator

Researcher Identifier: 0000-0003-1591-9389

Nearest person month worked: 4

Contribution to Project: Responsible for overall functioning of this portion of the project.

Funding Support: Federal Government Employee (Department of Defense)

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, protein expression investigations, and statistical analysis of data.

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: Vernieda Vergara, BS
Project Role: Research Assistant
Nearest person month worked: 12
Contribution to Project: Responsible for cell culture maintenance and metal analysis.
Note: Ms. Vergara was with the project from October 2016 to August 2018.

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 and departed in September 2020.

• Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report.

• What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

• COLLABORATIVE AWARDS:

Not Applicable.

• QUAD CHARTS:

Not Applicable.

9. APPENDICES:

Appendices

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Abbreviations Used

Al	Aluminum
BBB	Blood Brain Barrier
BMEC	Brain Microvascular Endothelial Cells
Co	Cobalt
Cu	Copper
DEET	N,N-Diethyl-m-toluamide
DMSO	Dimethylsulfoxide
DU	Depleted uranium
Fe	Iron
LDH	Lactate dehydrogenase
Mn	Manganese
Ni	Nickel
NR	Neutral Red
PB	Pyridostigmine bromide
PM	Permethrin
Sr	Strontium
TEER	Trans-endothelial electrical resistance
Zn	Zinc

Effect of Gulf War “Desert-Dust” compounds on the viability and permeability of the blood- brain barrier in a 3D model

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² Daniel K. Inouye Graduate School of Nursing, Uniformed Services University, Bethesda, MD

Gulf War Illness (GWI) refers to the chronic multi-symptom illness characterized by cognitive problems, fatigue, and muscle pain suffered by over one-third of American veterans who served in the Persian Gulf War in 1990-1991. Investigations into potential causes suggest a multiple exposure scenario, possibly to a combination of the nerve gas prophylactic pyridostigmine bromide (PB), insecticide N,N-diethyl-*m*-toluamine (DEET), and pesticide permethrin (PM), rather than a single exposure incident. Experiments have shown combining exposures to PB with PM or DEET disrupts the blood-brain barrier (BBB) and causes neurological and behavioral deficits, but does not account for all reported symptoms of GWI. Another study suggests PB alone can increase BBB permeability, allowing a virus to cross over into the brain where it is not normally found. Other studies suggest that respiratory exposure to the fine-grained sand particles found in the area, deemed “desert dust,” could also be linked to GWI. Analysis of this desert dust found high levels of a variety of metals, including aluminum, iron, uranium, nickel, cobalt, copper, strontium, manganese, and zinc. Under certain conditions, metals have been known to cross the BBB into the brain and induce neuronal injury and behavioral changes.

We hypothesize that combined exposures of PB, PM, and DEET adversely affect BBB permeability, allowing metals solubilized from inhaled desert dust particles to enter the brain. To test this, we used a combination of traditional cell culture and a 3D dynamic *in vitro* model of the BBB (DIV-BBB). First, human brain endothelial cells (Cell Systems, ACBRI 376) and human astrocytes (Lonza, CC-2565) were individually exposed to PB, PM, DEET, nickel, cobalt, strontium, zinc, manganese, copper, iron, aluminum, or depleted uranium (dose range 0.1 to 1000 μ M) and assessed for viability and function using standard cell culture techniques. For most compounds, we found 1 μ M to be a sufficiently sub-toxic dose, which was then used in the 3D DIV-BBB system (FloCel). Endothelial cells in the luminal (blood) side of a 3D cartridge were co-cultured with astrocytes in the abluminal (brain) side of the cartridge, and allowed to establish tight junctions evidenced by a high transendothelial electrical resistance (TEER) value. The closed system, which provided a more realistic model of blood flow and metabolite exchange across a BBB, was exposed to sub-toxic levels of PB, PM, and DEET for 24 hours, followed by sub-toxic levels of each metal and monitored for changes in TEER and the translocation of metal from the luminal to abluminal side.

Funding: CDMRP Gulf War Illness Research Program: W81XWH-16-2-0050

The views expressed in this presentation are those of the authors and do not reflect the official policy or position of the Armed Forces Radiobiology Research Institute, the Uniformed Services University, the Department of Defense, or the United States Government.

Effects of co-exposure to organic compounds and “desert dust” metals on the integrity of the blood brain barrier: a possible cause of Gulf War Illness

Jessica F. Hoffman¹, Anya X. Fan¹, Vernieda B. Vergara¹, John F. Kalinich^{1*}

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GWIRP Award: W81XWH-16-2-0050

Research Topic: Pathobiology/Pathophysiology

Hypothesis: The range of symptoms and large number of personnel experiencing Gulf War Illness do not all fit known single exposure scenarios, suggesting instead a multiple exposure scenario. Many military personnel were exposed to multiple pesticides and nerve agent prophylactics as well as inhaled metal and other particulates in the fine desert dust of the area occupied during the Gulf War. We hypothesize that exposures of pyridostigmine bromide (PB), permethrin (PM), or N,N-diethyl-3-methylbenzamide (DEET) adversely affect blood-brain barrier (BBB) permeability, allowing metals solubilized from inhaled desert dust particles to enter the brain and induce oxidative damage.

Methodology: To test this, we assessed viability and function of human neural cells after individual or concurrent exposure to the organic compounds (PB, PM, DEET), and multiple metals identified in desert dust using *in vitro* cell cultures of brain microvascular endothelial cells (BMECs) and astrocytes individually and in a transwell co-culture BBB model. Using sub-toxic concentrations of the organic and metal compounds, the integrity of the BBB was assessed by measuring changes in BBB protein expression, trans-epithelial electrical resistance (TEER), release of inflammatory cytokines, and induction of oxidative damage (lipid peroxidation, protein carbonyl formation, production of reactive nitrogen species). In addition, the ability for metal ions to cross from one side of the BBB to the other, as measured by inductively coupled plasma-mass spectroscopy, was also assessed.

Results: As expected, increasing doses of organic compounds and metals were increasingly toxic to both endothelial and astrocyte cell cultures. Using cell toxicity and mitochondrial viability assays we determined the sub-toxic dose for most organics was 10 μ M, and 1 μ M for most metals. Using sub-toxic concentrations of PB, PM, or DEET in a transwell BBB model, it was found that both PB and PM, but not DEET, affected TEER. Further, PB treatment decreased expression of occludin and zonula occludens (ZO-1) in BMECs.

Conclusions: The results of this project thus far indicate that pesticides and prophylactics, PB in particular, can affect the BBB and disrupt the expression of proteins required for tight junction formation.

Research supported by grant W81XWH-16-2-0050 of the Gulf War Illness Research Program.

GWIRP Award: W81XWH-16-2-0050

Research Topic: Pathobiology/Pathophysiology

Effects of co-exposure to organic compounds and “desert dust” metals on the integrity of the blood brain barrier: a possible cause of Gulf War Illness

Jessica F. Hoffman, Anya X. Fan, Vernieda B. Vergara, John F. Kalinich

Internal Contamination and Metal Toxicity Program



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Disclosure

The views expressed in this presentation are my own and do not reflect the official policy or position of the Armed Forces Radiobiology Research Institute, Uniformed Services University, Department of Defense, or US Government.

The authors have no conflicts of interest to report.

Background

- U.S. military personnel in the 1990-1991 Gulf War were exposed to a variety of chemical compounds including insecticides, pesticides, and a prophylactic treatment for nerve agent exposure.
- Personnel in theater were also exposed daily to the fine-grained sand in the area. U.S. Army Public Health Command estimated that particulate levels exceeded Military Exposure Guidelines over 97% of the time.
- Because of the small size of the particulates, it is often called “desert dust”. Analysis of the material showed that these dust particles possessed a clay or quartz core surrounded by an inorganic layer of calcium carbonate containing a wide variety of metals including, but not limited to, aluminum, iron, uranium, cobalt, copper, nickel, strontium, and manganese.

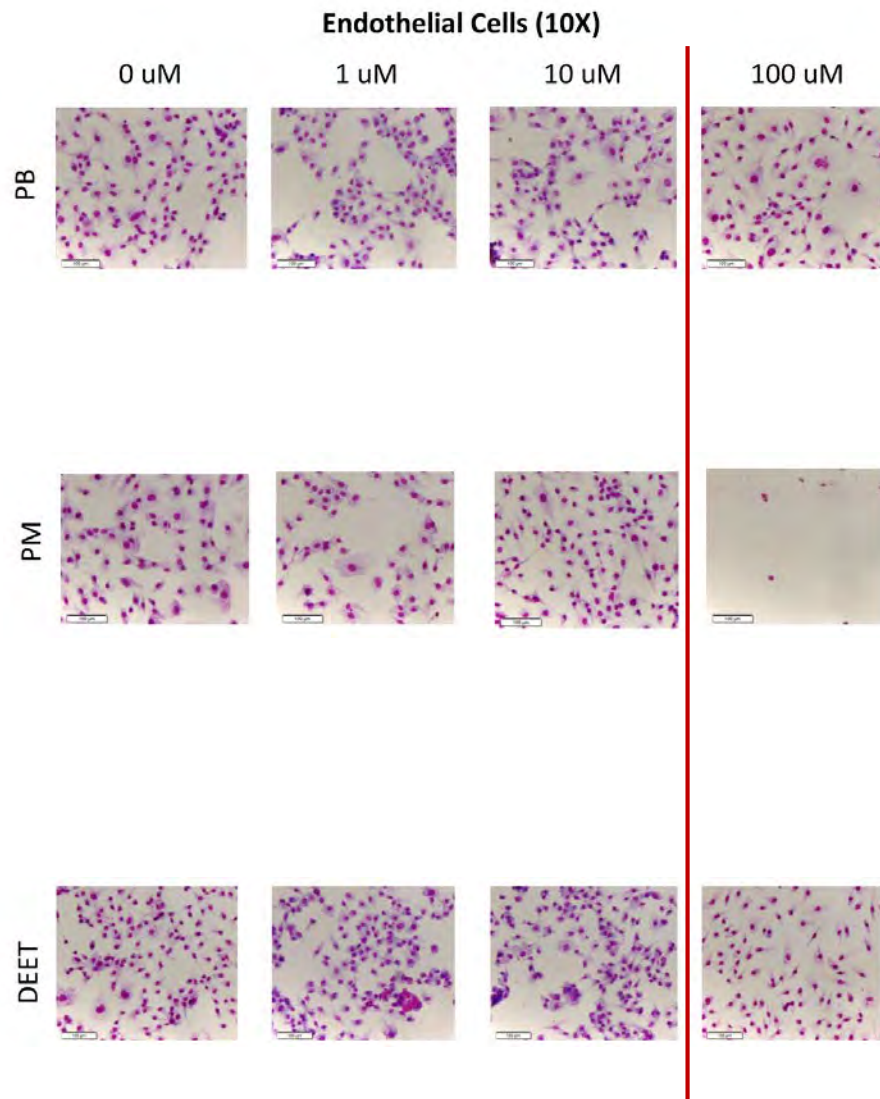
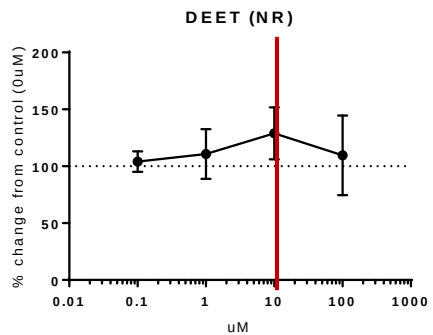
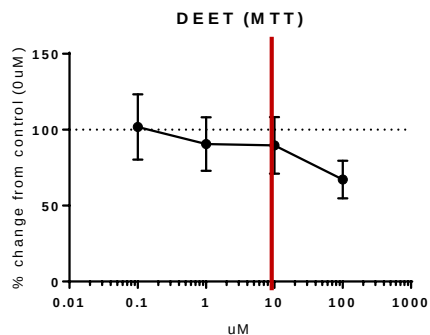
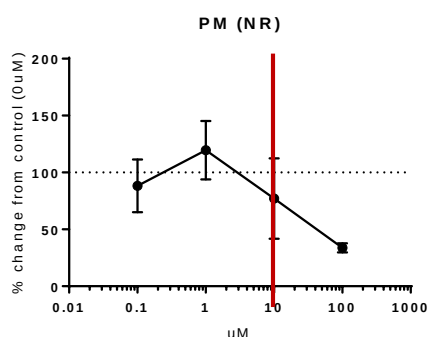
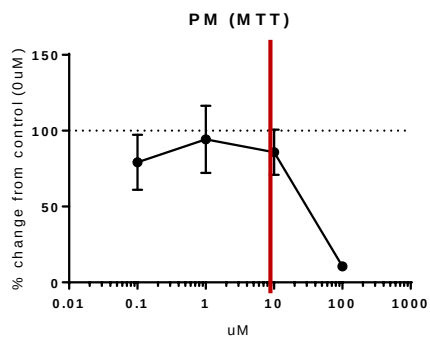
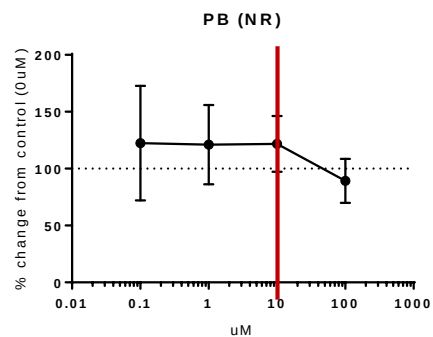
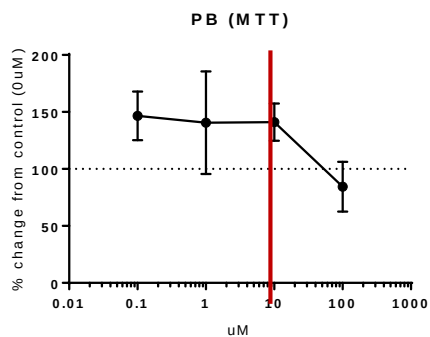
Hypothesis

- GWI is due to a multi-exposure scenario where exposure to pyridostigmine bromide (PB), permethrin (PM), or N,N-diethyl-3-methylbenzamide (DEET), adversely affects blood-brain barrier (BBB) permeability, allowing metals solubilized from inhaled desert dust particles to enter the brain and induce oxidative damage.

Methods

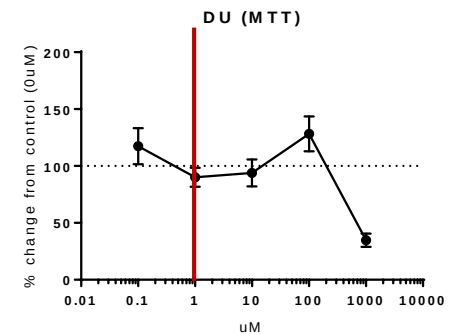
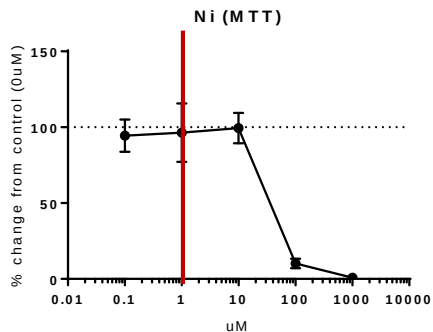
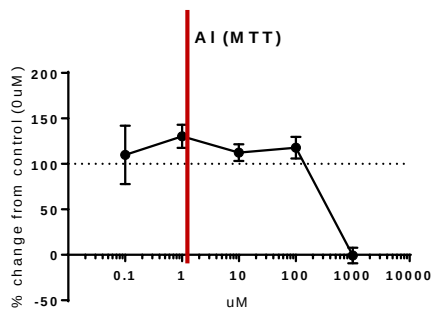
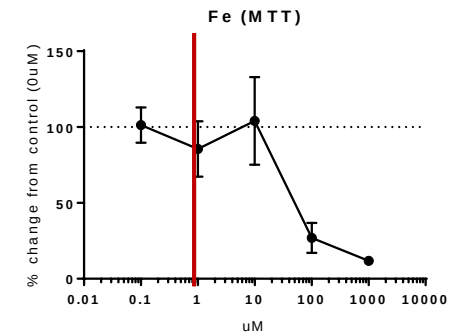
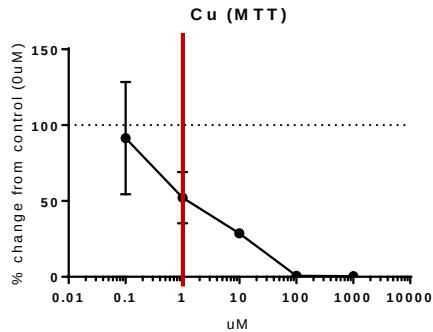
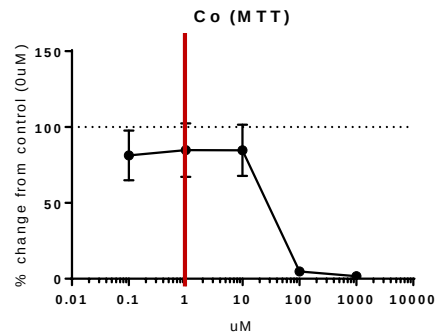
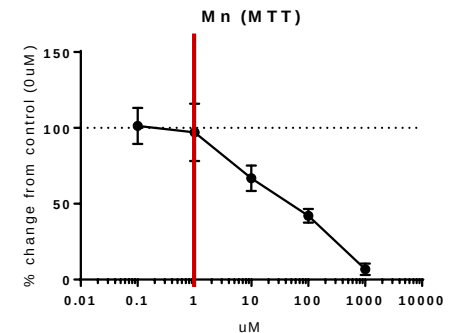
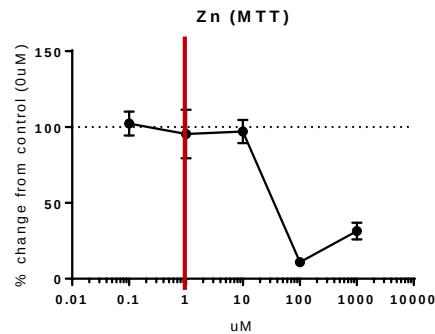
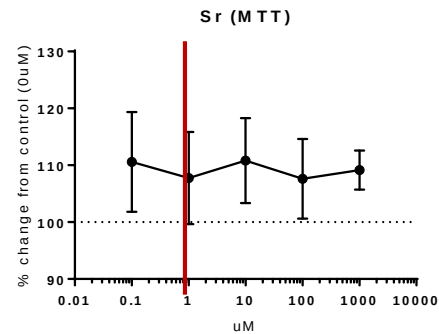
- Assess viability and function of brain microvascular endothelial cells (BMEC) and astrocytes in multi-exposure scenarios.
 - Determine sub-toxic level of organics and metals alone.
 - Assess integrity and function of an in vitro BBB model after organic and metal exposure.
 - Investigate induction of oxidative stress damage and changes in protein expression as a result of organic and metal exposure.

Organic Compounds - Dose Response on Brain Endothelial Cell Viability



10 μ M chosen as sub-toxic dose for organics

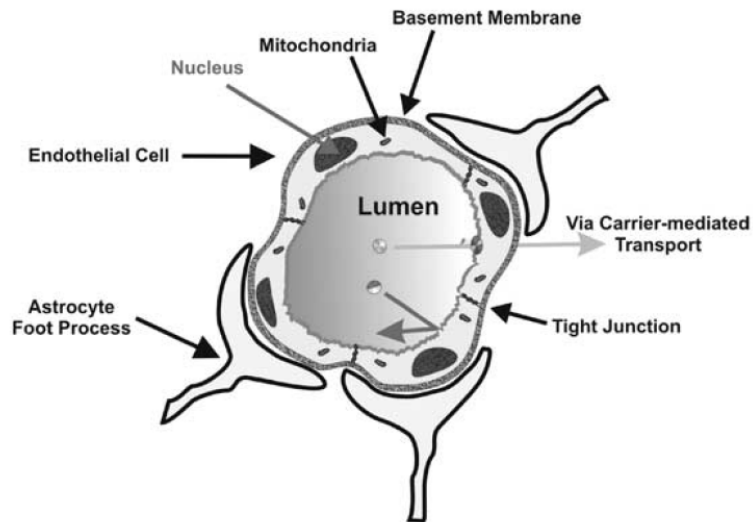
Metal Compounds - Dose Response on Brain Endothelial Cell Viability



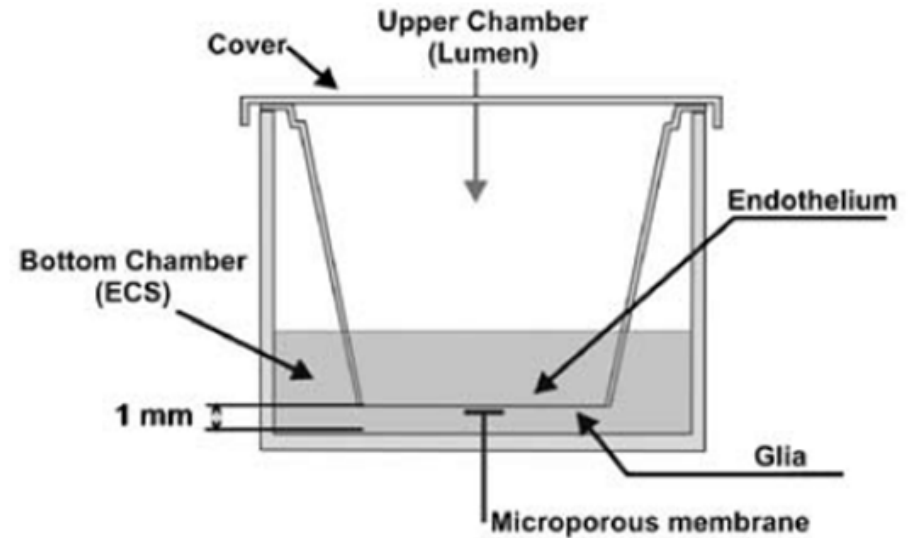
1 μ M chosen as sub-toxic dose for metals

The Blood Brain Barrier

In vivo BBB scheme

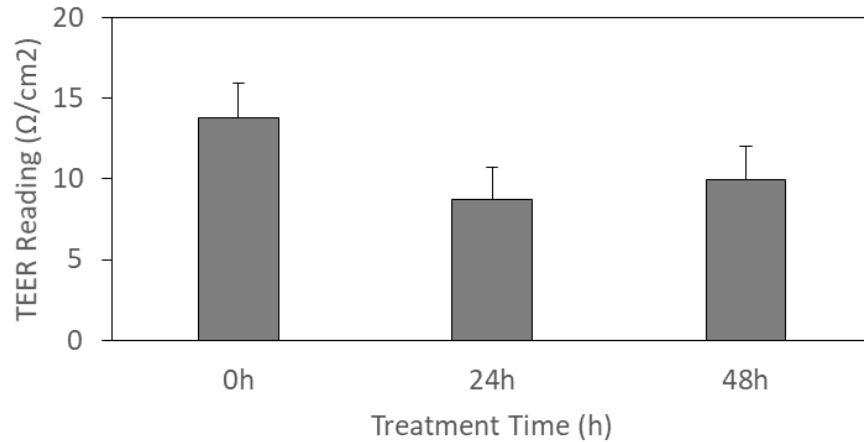


Transwell

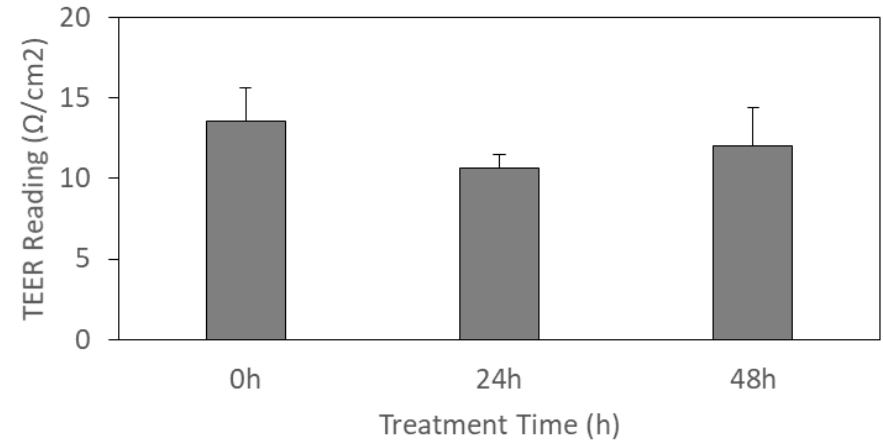


Effect of Organics on BBB TEER

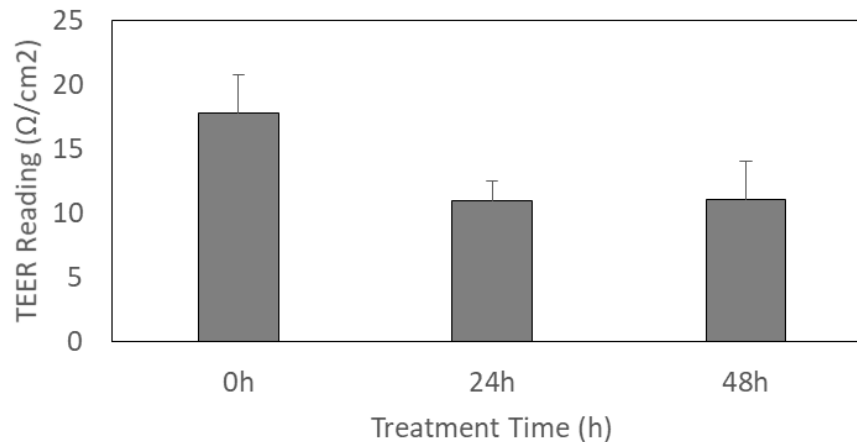
Effect of PB Treatment on TEER Values



Effect of DEET Treatment on TEER Values

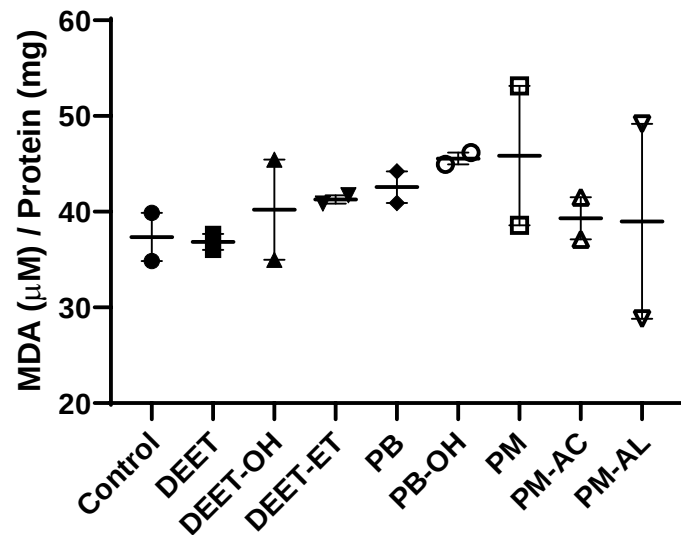


Effect of PM Treatment on TEER Values

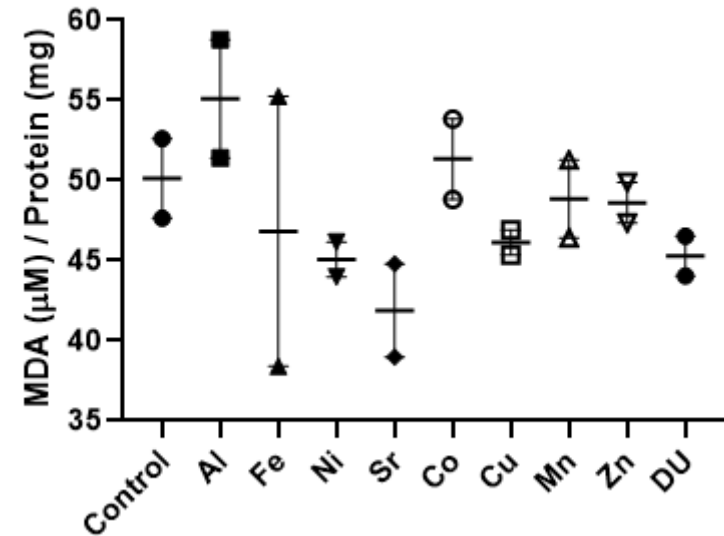


Effects of organics plus metals on BMEC oxidative damage

TBARS - single exposure organics

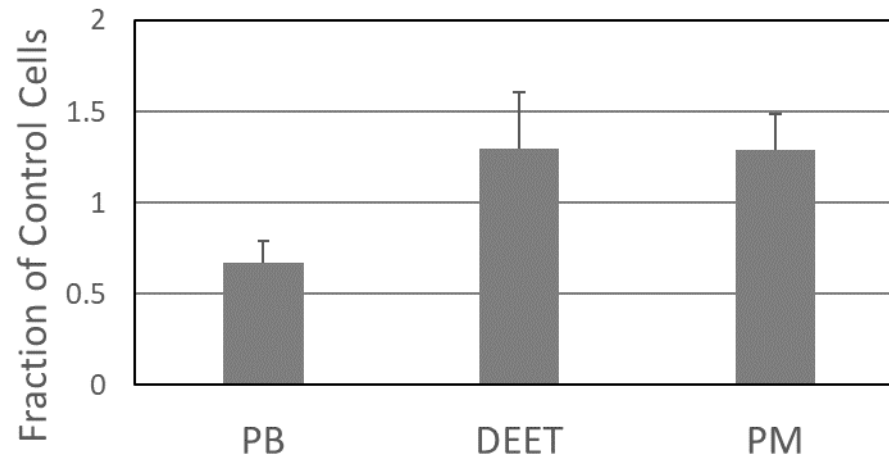


TBARS - single exposure metals

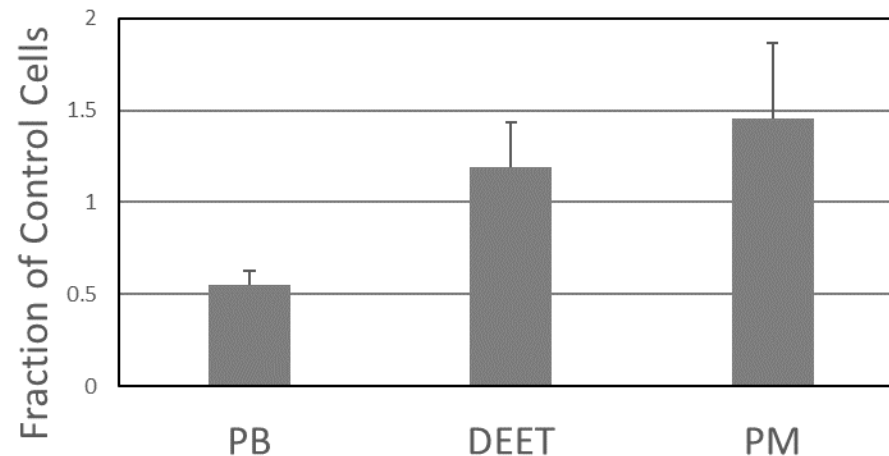


Protein Expression in Treated Brain Microvascular Endothelial Cells

ZO-1 Levels in Treated BMECs



Occludin Levels in Treated BMECs



Conclusions

- Since the project is not yet completed, no definitive conclusions can be offered.
- Toxicological assessments of PM, PB, and DEET, as well as nine different metals found in desert dust, were conducted on human brain microvascular endothelial cells and human astrocytes.
- Using an in vitro blood brain barrier model system consisting of these two cell lines, it was observed that treatment with sub-toxic concentrations of PB or PM caused a reduction in TEER values in a time-dependent manner. Treatment with DEET initially resulted in a decrease in TEER but returned to near-normal levels over time.
- Studies to assess the induction of oxidative damage after organic and metal exposure are currently underway.
- Treatment with PM, PB, or DEET was found to affect expression of ZO-1 and occludin in treated BMECs.



Article

Effects of Incubation of Human Brain Microvascular Endothelial Cells and Astrocytes with Pyridostigmine Bromide, DEET, or Permethrin in the Absence or Presence of Metal Salts

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Abstract: Gulf War Illness (GWI) is a chronic, multi-symptom illness suffered by over one-third of American military veterans who served in the Persian Gulf War between 1990 and 1991. No current single-exposure scenario accounts for all the symptoms observed in GWI, and instead may be due to a multi-exposure scenario. As a larger effort to understand how one category of multi-exposure scenarios of organic compounds such as nerve gas prophylactic pyridostigmine bromide, or insecticides/pesticides such as N,N-diethyl-*m*-toluamide (DEET) and permethrin, plus heavy metals found in inhaled dust particles (Al, Fe, Ni, Sr, DU, Co, Cu, Mn, and Zn) might play a role in neural aspects of GWI, we begin this initial study to examine the toxicity and oxidative damage markers of human brain endothelial cell and human astrocyte cell cultures in response to these compounds. A battery of cytotoxicity assessments, including the MTT assay, Neutral Red uptake, and direct microscopic observation, was used to determine a non-toxic dose of the test compounds. After testing a wide range of doses of each compound, we chose a sub-toxic dose of 10 μ M for the three organic compounds and 1 μ M for the nine metals of interest for co-exposure experiments on cell cultures and examined an array of oxidative stress-response markers including nitric oxide production, formation of protein carbonyls, production of thiobarbituric acid-reactive substances, and expression of proteins involved in oxidative stress and cell damage. Many markers were not significantly altered, but we report a significant increase in nitric oxide after exposure to any of the three compounds in conjunction with depleted uranium.

Keywords: Gulf War Illness; pyridostigmine bromide; permethrin; DEET; desert dust; metal; toxicity

1. Introduction

Gulf War Illness (GWI) refers to the chronic multi-symptom illness characterized by cognitive problems, fatigue, and muscle pain suffered by over one-third of American veterans who served in the Persian Gulf War in the period 1990–1991—close to 700,000 U.S. military personnel. A variety of exposures have been proposed as potential factors including nerve gas, vaccinations/prophylactics, depleted uranium, smoke from oil-well fires, stress, pesticides, and insecticides. However, the large number of personnel experiencing this multi-symptom illness did not all fit known single-exposure scenarios, suggesting instead a multi-exposure scenario. A review by [1] provides a good overview of the history and numerous studies around GWI.

Pyridostigmine bromide (PB), originally approved by the Food and Drug Administration (FDA) for treatment of myasthenia gravis, was given investigational status as a nerve gas prophylactic for the First Gulf War, but was not recommended for routine use. The requirement for informed

consent and extensive record keeping was waived for the Department of Defense (DoD), however, and personnel were given blister packs of PB tablets and instructed to self-administer every 8 h [2,3]. Approximately a quarter-million personnel received at least one dose [4], but actual dose exposure to individuals is unknown. The U.S. Department of Agriculture (USDA) developed N,N-diethyl-*m*-toluamide (DEET) as an insecticide/insect repellent for the military in the 1940s, and it has had wide annual use [5]. DEET is rapidly absorbed by the skin and there are concerns that it could increase the uptake of other chemical, metal, or viral contaminants on the skin [6,7]. 3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarboxylic acid, known as permethrin (PM), is a synthetic pesticide used as an aerosol spray to treat the clothing and living spaces of military personnel during the Gulf War. Proper use is connected with no or minor adverse effects [8–11], but it can be toxic at high exposure levels, at least in animals [12,13].

Several studies have suggested that PB alone or in combination with other exposures, including stress, DEET, or PM, could be linked with illnesses in Gulf War veterans [14–22]. One study reported that treatment of mice with PB prior to virus exposure enabled the virus to cross the blood–brain barrier (BBB) and enter the brain, suggesting that although PB alone could not cross the BBB, it could increase the permeability of the barrier to other compounds [23]. Other studies report disruption of the BBB and significant damage in specific areas of the brain [24–26] as well as neurological deficits [27–30].

Other studies suggest that respiratory exposure to the fine-grained sand particles found in the area, deemed “desert dust,” could also be linked to GWI [31,32]. Analysis of this desert dust found high levels of a variety of metals, including aluminum, iron, uranium, nickel, cobalt, copper, lead, chromium, strontium, tin, manganese, zinc, barium, arsenic, and vanadium, as well as microbial contaminants [33–35]. The U.S. Army Center for Health Promotion and Preventive Medicine (now the U.S. Army Public Health Command) estimates that during Operation Iraqi Freedom and Operation Enduring Freedom, particulate levels exceeded the Military Exposure Guidelines (MEG) over 97% of the time [36]. There is growing evidence that metals internalized through ingestion, inhalation, or wounds, cross the BBB and induce neuronal injury, behavioral changes, and development of neurological diseases [37–46]. Once accumulated in the brain, many metals induce oxidative stress and alter essential trace metal pools in various regions of the brain, further affecting neurological function [47–49].

Our overall hypothesis is that combined exposures of PB, PM, and DEET adversely affect BBB permeability, allowing metals solubilized from inhaled desert dust particles to enter the brain and result in extensive oxidative damage. In order to begin testing this hypothesis and establish an *in vitro* BBB model system, we first assessed viability and function of human neural cells after individual or concurrent exposure to PB, PM, DEET, and multiple metals identified in desert dust using *in vitro* cell cultures of human brain microvascular endothelial cells (BMECs) and human astrocytes and report those results herein.

2. Materials and Methods

2.1. Cells and Cell Culture

2.1.1. Human Astrocytes

Normal human astrocytes (male, CC-2565, Lonza, Walkersville, MD, USA) were used between passages 2 and 6 for the initial toxicity studies. However, because of vendor delays, further studies were completed using normal human astrocytes (male, #1800) from ScienCell Research Labs (Carlsbad, CA, USA). Cells were maintained in Dulbecco’s Minimal Essential Medium (D-MEM) with Glutamax (Gibco, Grand Island, NY, USA) supplemented with N2 Supplement CTS (Gibco) and Fetal Bovine Serum One-Shot (10%, Gibco) and were not used past passage 8 for all other experiments.

2.1.2. Human Brain Microvascular Endothelial Cells

Human brain microvascular endothelial cells (male, ACBRI 376) were obtained from Cell Systems (Kirkland, WA, USA) and cultured in Vasculife VEGF Basal Medium supplemented with the associated VEGF LifeFactors Kit (Lifeline Cell Technology, Frederick, MD, USA) with the following modifications. The LifeFactors Kit fetal bovine serum was not used and only 10 mL of the LifeFactors-provided L-glutamine was used as a supplement. In addition, iCell Endothelial Cell Medium Supplement (Cellular Dynamics International, Madison, WI, USA) was added to 10%. Cells were used between passages 3 and 6 for the initial toxicity studies and not past passage 8 for all other experiments.

2.1.3. Culture Setup

Stock cell cultures were maintained in tissue culture flasks (75 cm² area) at 37 °C in a humidified atmosphere of 5% CO₂. For endothelial cells, the flasks were treated with 6 mL of Quick-Coat (Angio-Proteomie, Boston, MA, USA) to provide a suitable substrate for attachment. For both cell lines, medium was refreshed every other day and cells were subcultured when 70–80% confluent based upon direct microscopic observation. Briefly, medium was aspirated from the flasks and the flasks were washed once with phosphate-buffered saline (PBS, Gibco). After aspirating the PBS, 3 mL of StemPro Accutase Cell Dissociation Reagent (Gibco) were added and the flask left at room temperature for up to 8 min to allow the cells to dissociate from the flask. Once detached, fresh medium was added, and the cell suspension pipetted to break up any clumps. The cell suspension was centrifuged at 240× g for 10 min, after which the cell pellet was resuspended in a suitable amount of the appropriate medium and transferred to new flasks. Cell numbers and viability were determined using trypan blue (Gibco) exclusion with the Bio-Rad Model TC-20 Cell Counter (Hercules, CA, USA).

2.1.4. Fluorescent Immunohistochemistry of BMEC Markers

BMEC cells were cultured onto 8-chamber slides (Nunc LabTek II, Fisher Scientific, Pittsburgh, PA, USA) as described in Section 2.3.1, then fixed with 100% methanol chilled at −20 °C for 5 min and washed 3 times with ice cold PBS. Chambers designated for ZO-1 staining were permeabilized by incubation with 0.1% Triton X-100 in PBS for 10 min, then washed 3 times in 5 min washes of PBS. Chambers designated for occludin staining were not permeabilized. All chambers were then incubated with 1% BSA, 22.52 mg/mL glycine in PBST (PBS + 0.1% Tween20) for 30 min to block non-specific binding. Chambers were then incubated with 1% BSA PBST (without glycine) and the designated dilution of primary antibody ZO-1 or occludin overnight at 4 °C. Chambers were then decanted, washed 3× in PBS 5 min each, and incubated with appropriate secondary antibody in 1% BSA PBS for 1 h at room temp in the dark, followed by a decant and 3 washes of PBS, 5 min each. Chambers were then counterstained with 1 µg/mL DAPI for 1 min and rinsed with PBS. Chamber sides were removed, coverslipped with permount, and imaged as described in Section 2.3.1. Antibody information is presented in Supplementary Materials Table S1, and images of overlaid target antibody with DAPI are presented in Supplementary Materials Figure S1.

2.2. Organics and Metals for Treatment

2.2.1. Organic Compounds of Interest

The following compounds were tested, pyridostigmine bromide (PB), permethrin (PM), and N,N-diethyl-*m*-toluamide (DEET) (all from Sigma Chemical, St. Louis, MO, USA). Stock solutions were prepared in dimethylsulfoxide (DMSO, Invitrogen, Carlsbad, CA, USA) before dilution to appropriate working concentrations in cell culture medium.

2.2.2. Metals of Interest

Metals to be tested included aluminum (Al as aluminum chloride hydrate), iron (Fe as iron chloride hexahydrate), uranium (depleted uranium as uranyl nitrate hexahydrate), nickel (Ni as nickel chloride hexahydrate), cobalt (Co as cobalt chloride hexahydrate), copper (Cu as copper chloride dihydrate), strontium (Sr as strontium chloride hexahydrate), manganese (Mn as manganese chloride tetrahydrate), and zinc (Zn as zinc chloride). All metals were obtained from Sigma Chemical except for uranyl nitrate hexahydrate which was from Fluka Chemical (Ronkonkoma, NY, USA). Metals were dissolved in the appropriate cell culture medium and diluted to provide proper working concentrations.

2.3. Viability and Toxicity Assays to Determine Sub-toxic Exposure Dose

2.3.1. Cell Culture

Cells were plated onto 96-well plates at 5000 cells/well (astrocytes) or 2500 cells/well (BMEC) 100 μ L per well, and allowed to incubate at 37 °C, 5% CO₂ for 24 h. Media was then removed and cells treated with media spiked with metal or organic compounds in a serial dose dilution ranging from 0 μ M to 1000 μ M and incubated for an additional 24 h prior to viability assays. For images, cells were plated at 50,000 cells/chamber on Nunc Lab-Tek II 8-chamber slides, treated in the same manner as the plates. Medium was aspirated, the chambers washed with Dulbecco's PBS (DPBS, Gibco) and cells fixed with ice-cold methanol for 5 min. After removing the methanol, the slides were air dried before being stained with Giemsa (Gibco) stain (1:20 dilution of stock) for 10 min followed by extensive washing with tap water. Images were taken with an Olympus BX61 Microscope with D72 camera and cellSens Entry Software (version 1.5) (Olympus America, Melville, NY, USA).

2.3.2. MTT Assay

Metabolic viability was assessed using the CellTiter 96[®] Aqueous One Solution Cell Proliferation Assay kit (Promega Corporation, Madison, WI, USA). The assay is based upon the ability of dehydrogenase enzyme systems, located in the cell mitochondria, to reduce a tetrazolium compound to a colored formazan product, which is easily detected colorimetrically. Briefly, after the 24 h treatment incubation period, 20 μ L of CellTiter 96[®] Aqueous One Solution Reagent was added to each plate well, incubated 1 h, then absorbance determined at 490 nm using a microplate reader (SpectraMax Model 250 Microplate Spectrophotometer, Molecular Devices Corporation, Sunnyvale, CA, USA). Metabolic viability of the treated cells was normalized to the media-only control cells.

2.3.3. Neutral Red (NR)

Cell toxicity was assessed using the In Vitro Toxicology Assay Kit (Sigma Chemical). Neutral Red dye (Toluylene Red) is only taken up by viable cells via active transport, thus a decrease in the amount of dye released from cell culture indicates more cytotoxic conditions. Briefly, after the 24 h treatment incubation period, 10 μ L Neutral Red dye was added to each plate well, incubated for 2 h, washed twice with 100 μ L DPBS, and incubated at room temperature for 10 min with 100 μ L Stabilizing Solution in each well for 10 min prior to determining absorbance at 540 nm using a microplate reader. Viability of the treated cells was normalized to the media-only control cells.

2.4. BMEC Culture Experiments for Protein Expression and Oxidative Damage Assessments

The following procedure was used to provide samples to determine protein expression and assess oxidative damage of endothelial cells treated with metals in the presence or absence of Gulf War-associated organic compounds. The basic experimental plan was to plate the cells on Day 1, replace the medium on Day 2, add organic compounds (if required) on Day 3, add metals (if required) on Day 4, and harvest on Day 5. Briefly, BMECs were harvested as above and re-plated on 6-well tissue culture plates at a concentration of 2×10^5 cells/well and the plates returned to the incubator. The next

day the medium was aspirated and replaced with fresh medium (2 mL/well). If required, on the next day the appropriate organic compound was added to a non-toxic dose (as determined above) and the plates returned to the incubator. The next day, if required, appropriate metals were added, again at a non-toxic dose as previously determined and the plates returned to the incubator. The next day, 1 mL of medium was removed from each well, placed in a 1.5 mL tube and centrifuged at $18,400\times g$ for 10 min to pellet any detached cells. The supernatant (0.8 mL) was then removed, placed in a fresh 1.5 mL tube and stored at $-80\text{ }^{\circ}\text{C}$ until needed for the nitric oxide assay. The remaining medium in the wells was aspirated and the wells washed with 2 mL of PBS (Gibco). After aspirating the PBS, 1 mL of Accutase was added to each well and the plates incubated at room temperature for 8 min to detach the cells. The cell suspension was placed in a 1.5 mL tube and centrifuged at $18,400\times g$ for 10 min to pellet the cells. After removing the supernatant, the cell pellets were stored at $-80\text{ }^{\circ}\text{C}$ until required for protein expression analysis and oxidative damage assessments.

2.5. Reactive Nitrogen Species

The Nitrate/Nitrite Colorimetric Assay Kit (#78001, Cayman Chemical, Ann Arbor, MI, USA) was used to analyze nitric oxide (NO) levels in the collected cell culture supernatants. The final products of nitric oxide are nitrate and nitrite, but since the relative proportions of these compounds can be variable, the best assessment of total nitric oxide production is the sum of both of these. This is accomplished by converting the nitrate in the sample to nitrite using nitrate reductase, then converting the nitrite to an azo compound using the Griess reaction. Briefly, 80 μL of cell supernatant was mixed with 10 μL of enzyme cofactor mixture and 10 μL of nitrate reductase mixture in a 96-well plate. The plate was incubated at room temperature for 1 h, after which the Griess reagents provided in the kit were added to each well. After 10 min at room temperature, absorbance was read at 540 nm in a BioTek Synergy Model H1M Multimodal Plate Reader with GEN5 Software (BioTek Instruments, Winooski, VT, USA) and the amount of nitrate determined from a standard curve and expressed in μM .

2.6. Lipid Peroxidation (TBARS) Assay

The effect of the various treatments on the induction of lipid peroxidation was determined using the thiobarbituric acid-reactive substances (TBARS) assay from Cayman Chemical (kit #10009055). The stored cell pellets collected from the 6-well plates were resuspended in 200 μL of PBS and homogenized with a sonicator (Fisherbrand Model 120 Sonic Dismembrator, Pittsburgh, PA, USA) for 5 s with a 1 s pulse at 40% amplitude. Total protein from each sample supernatant was measured by Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA, cat #500-0006), in duplicate, against a BSA standard curve, on a spectrophotometer (BioTek Synergy Model H1M Multimodal Plate Reader with GEN5 Software), and read at 595 nm. Briefly, 100 μL of cell homogenate was mixed with 100 μL of TCA Assay Reagent and 800 μL of Color Reagent (both provided in the kit) and the tubes placed in a heat block at $95\text{ }^{\circ}\text{C}$ for 60 min. They were then placed on ice for 10 min before centrifugation for 10 min at $1600\times g$. Supernatant ($2\times 100\text{ }\mu\text{L}$ aliquots) were removed and placed in a black plate for fluorescence measurement using 530 nm excitation and 550 nm emission in a BioTek Synergy Model H1M Multimodal Plate Reader with GEN5 Software. The amount of thiobarbituric acid-reactive substance in the samples was quantitated using a standard curve prepared with the kit-provided TBA Malondialdehyde Standard with results normalized to protein content.

2.7. Protein Carbonyl Levels

Oxidative damage to protein side chains was assessed using the protein carbonyl assay. The Abcam Protein Carbonyl Content Fluorometric Assay Kit (ab235631, Abcam, Cambridge, MA, USA) was used for this purpose. Cell homogenates (50 μL), prepared as above, were added to 50 μL FTC fluorophore dissolved in Protein Carbonyl Assay Buffer (both provided in the kit) in a 1.5 mL tube. The tube was mixed and allowed to sit overnight at room temperature in the dark. Next, 200 μL of ice-cold 20% trichloroacetic acid was added and the tubes left on ice for 10 min. After this the tubes were centrifuged

at 10,000× g for 10 min, the supernatant removed, and the resulting pellet washed 3× with 200 µL of 100% isopropanol. After the last wash step, the tubes were allowed to dry at room temperature for 1 h. The pellet was resuspended in 50 µL of 6 M guanidine and heated at 50 °C for 2 h. After cooling to room temperature, 450 µL of Sample Dilution Buffer from the kit was added. Two 100 µL aliquots were taken and placed in a black 96-well plate for fluorescence measurement at 485 nm excitation and 535 nm emission in a BioTek Synergy Model H1M Multimodal Plate Reader with GEN5 Software. The amount of protein carbonyls in the samples was determined by comparing to a standard curve prepared from the FTC fluorophore. In addition, the amount of protein contained in the processed sample, after addition of the Sample Dilution Buffer, was determined using bicinchoninic acid (BCA Protein Assay Kit (#23277), Pierce Biotechnology, Rockford, IL, USA). Protein carbonyl values were normalized to protein content.

2.8. Protein expression using the ProteinSimple Wes

Cell pellets were re-suspended in RIPA buffer (Thermo, Waltham, MA, USA, cat#89901) plus Halt Protease and Phosphatase Inhibitor Cocktail (Thermo cat#78442) and homogenized in a Bullet Blender (Next Advance, Troy, NY, USA) with 0.5 mm glass beads (NextAdvance, cat#GB05-RNA) (settings speed 6, 5 min × 2 runs) and then centrifuged at 1340× g for 10 min. Total protein from each sample supernatant was measured by Bio-Rad Protein Assay (Bio-Rad Laboratories, cat #500-0006), in triplicate, against a BSA standard curve, on a spectrophotometer (BioTek Synergy Model H1M Multimodal Plate Reader with GEN5 Software), and read at 595 nm. Proteins of interest were quantified using an automated capillary-based size sorting chemiluminescent system 'WES' from ProteinSimple (San Jose, CA, USA). All procedures were performed with manufacturer's reagents (12-235 kDa kit, cat#SM-W004-1 and PS-ST01EZ-8) according to the user manual with some adjustments: samples are aliquoted to 1 µg/µL before mixing 4 µL with 1 µL fluorescent master mix, then denatured at 94 °F (34.4 °C) for 4 min, given a quick spin, and loaded on the plate at 4 µL per well. In run settings, stacking time was changed to 18 s, separation time to 31 min, and antibody diluent time to 30 min. Antibody information is listed in Supplementary Materials Table S1. Peak values were determined using Compass Software (ProteinSimple). Target proteins are presented as a ratio of the target protein expression normalized to within-sample β-actin expression in arbitrary units. Since the ProteinSimple Wes system uses capillary size separation and chemiluminescent detection, an example of the raw data run for one of the GPX4 DEET + metal experiment sample sets is shown in Supplementary Materials Figure S6a, and a traditional Western blot image derived from that data is in Supplementary Materials Figure S6b.

2.9. Statistical Analyses

Data in the figures represent the mean and standard deviation of three independent experiments. Analyses were performed using GraphPad Prism Software (version 8.4.2, La Jolla, CA, USA). One-way ANOVAs with Sidak's multiple comparisons post-hoc test were performed, with specific group comparisons or any other statistical analyses noted. Specific post-hoc *p* values less than 0.05 were considered significant.

3. Results

We hypothesize that exposures of sub-toxic levels of DEET, PB, and PM, particularly in combination with concurrent exposure to sub-toxic levels of metals, adversely affect the health and viability of cells involved in the blood–brain barrier (BBB), which could lead to subtle changes in permeability and function. To test this, human brain endothelial cells and human astrocytes exposed to DEET, PB, and PM, nickel, cobalt, strontium, zinc, manganese, copper, iron, aluminum, or depleted uranium, individually or in combination, were assessed for toxicity and viability using standard cell culture techniques.

3.1. Determination of Sub-toxic Doses of Organic and Metal Compounds

We began by identifying a sub-toxic dose of each compound to be used in experiments to examine subtle changes in blood–brain barrier integrity rather than outright or extensive damage. Monocultures of BMECs or astrocytes were exposed to each organic or metal compound of interest in a dose range of 1–1000 μM and assessed for toxicity using MTT and NR assays. MTT measures metabolic activity, and measurements after treatment were normalized back to media-only controls and presented as percent of control, where 100% represents no change from control (0 μM compound) and indicated as a horizontal dashed line in graphs. Values below this line indicate reduced metabolic activity, and thus are considered toxic. Similarly, the same experimental design was conducted and assayed with Neutral Red, which measures cell viability. Measurements are also presented as percent of control, and values below this line indicate fewer viable cells, and thus are toxic doses. MTT and NR results for BMECs and astrocytes exposed to organic compounds are presented in Supplementary Materials Figure S2, MTT and NR results for BMECs exposed to metals are presented in Supplementary Materials Figure S3, and MTT and NR results for astrocytes exposed to metals are presented in Supplementary Materials Figure S4. Additionally, cells were also cultured on slides, exposed to the same compound dose ranges, and stained with Giemsa to visualize changes in cell structure (BMECs images are presented in Supplementary Materials Figure S5a,b, astrocyte images are presented in Supplementary Materials Figure S6a,b). No quantitative analyses were conducted on the images. As expected, increasing doses of organic compounds and metals were increasingly toxic to both endothelial and astrocyte cell cultures. For most organic compounds, the 10 μM concentration is at or near the line of no change from control while the 100 μM dose is a subtle to sharp drop below normal. There is more variation in toxicity patterns with the metals, but for most metals, the 1 μM concentration is at or near the line of no change from control while the 10 μM dose is a decrease from control for several metals, and 100 μM is a drastic decrease in viability for most metals. We decided that 10 μM would serve as the sub-toxic dose for organic compounds and 1 μM would serve as the sub-toxic dose for metals for experiments moving forward.

To determine whether there was any synergistic toxicity effects of co-exposures of organics and metals to BMECs, monocultures of BMECs were exposed to a media only control, 10 μM of an organic alone, or the organic in combination with one of the metals at 1 μM and assayed and analyzed via MTT test as above, where 100% represents no change from the media-only control. One-way ANOVAs were significant for all three experimental sets in Figure 1A, DEET + metals $F_{(9,20)} = 29.28$, $p < 0.0001$; Figure 1B, PB + metals $F_{(9,20)} = 21.17$, $p < 0.0001$; Figure 1C, PM + metals $F_{(9,20)} = 7.80$, $p < 0.0001$). None of the organic compounds alone were significantly different from control (one-sample t -test compared to 100, DEET $t = 1.32$, $df = 2$, $p = 0.32$; PB $t = 1.10$, $df = 2$, $p = 0.39$; PM $t = 1.58$, $df = 2$, $p = 0.26$). However, Co and Mn in conjunction with all three organic compounds were significantly more toxic to BMECs than the corresponding organic compound alone (Sidak post-hoc: DEET vs. DEET + Co * $p < 0.0001$; DEET vs. DEET + Mn * $p < 0.0001$; PB vs. PB + Co * $p < 0.0001$; PB vs. PB + Mn * $p < 0.0001$; PM vs. PM + Co * $p = 0.0310$; PM vs. PM + Mn * $p < 0.0001$).

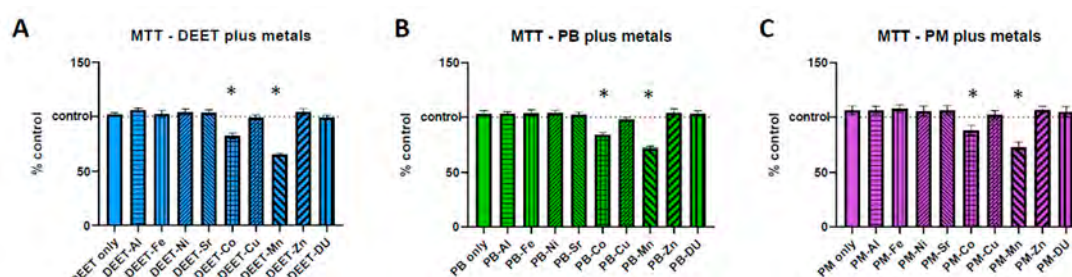


Figure 1. Toxicity of organic and metal combination exposures on brain microvascular endothelial cells (BMEC). Toxicity assessment via MTT assay on BMEC cultures for combinations of an each organic compound with individual metals. (A) DEET (10 μM) plus metals (1 μM), (B) pyridostigmine bromide (PB) (10 μM) plus metals (1 μM), and (C) permethrin (PM) (10 μM) plus metals (1 μM). Dotted line at 100 indicates 100% of control measurement: values below the line indicate that the dose is toxic to the cells and viability is decreased. Data represent the mean and standard deviation of three independent experiments. An * denotes a statistically significant difference from control at $p < 0.05$.

3.2. Nitric Oxide (NO) Production

(BMEC). Toxicity assessment via MTT assay on BMEC cultures for combinations of an each organic compound with individual metals. (A) DEET (10 μ M) plus metals (1 μ M), (B) pyridostigmine bromide (PB) (10 μ M) plus metals (1 μ M), and (C) permethrin (PM) (10 μ M) plus metals (1 μ M). Dotted line at 100 indicates 100% of control measurement: values below the line indicate that the dose is toxic to the cells and viability is decreased. Data represent the mean and standard deviation of three independent experiments. An * denotes a statistically significant difference from control at $p < 0.05$.

3.2. Nitric Oxide (NO) Production

NO increases in response to cell stress or damage. BMEC cell cultures were exposed to a media-only control, organic compounds and metals singly, or in sets of each organic alone and in combination with each metal. Within each experiment, NO signal was measured and normalized to the media-only control value and presented as percent of control, where 100% represents no change from control and indicated as a horizontal dashed line in graphs. Values above this line indicate an increase in NO concentration, and thus a status of more stressed or damaged cell culture. For each of the single exposure conditions (Figure 2A), a one-sample *t*-test of each % of control value is compared back to a value of 100 to determine if the treatment is different from the media-only control it was normalized to. Of all the organic compounds and metals alone, only DU was significantly different ($t = 30.80$, $df = 2$, $p = 0.0001$) (see Public Health 2020, 178).

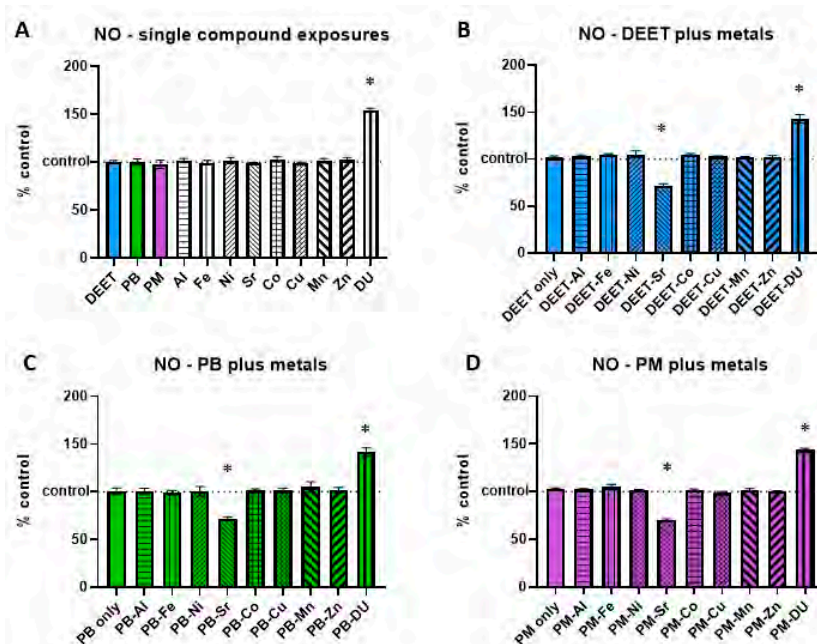


Figure 2. Nitric oxide (NO) in brain microvascular endothelial cell (BMEC) cultures after organic compound and metal exposure. Assessment of nitric oxide (NO) levels in BMEC cultures after exposure to organic compounds and/or metals. (A) organic compounds (10 μ M) or metals (1 μ M) alone, (B) DEET (10 μ M) plus metals (1 μ M), and (C) pyridostigmine bromide (PB) (10 μ M) plus metals (1 μ M) or (D) permethrin (PM) (10 μ M) plus metals (1 μ M). Dotted line at 100 indicates 100% of control measurement; values above the line indicate an increase in NO in the culture. Data represent the mean and standard deviation of three independent experiments. An * denotes a statistically significant difference from control at $p < 0.05$.

In the DEET or DEET + metals exposure experiment set (Figure 2B), DEET alone was not significantly different compared to media control (a one-sample t -test vs. 100%, $t = 0.97$, $df = 3$, $p = 0.40$). In combination with Cu, Ni, and Cr, DEET alone was significantly different from media control ($p = 0.0001$, $p = 0.0001$, and $p = 0.0001$, respectively). In the DEET + metals exposure experiment set (Figure 2B), DEET alone was not significantly different compared to media control (a one-sample t -test vs. 100%, $t = 0.97$, $df = 3$, $p = 0.40$). In combination with Cu, Ni, and Cr, DEET alone was significantly different from media control ($p = 0.0001$, $p = 0.0001$, and $p = 0.0001$, respectively). In the DEET + metals exposure experiment set (Figure 2B), DEET alone was not significantly different compared to media control (a one-sample t -test vs. 100%, $t = 0.97$, $df = 3$, $p = 0.40$). In combination with Cu, Ni, and Cr, DEET alone was significantly different from media control ($p = 0.0001$, $p = 0.0001$, and $p = 0.0001$, respectively).

DEET + Sr was significantly lower in NO production than DEET alone ($* p < 0.0001$), indicating a possible protective effect of Sr.

The PB or PB + metals exposure experiment set (Figure 2C, $F_{(9,30)} = 23.20$, $p < 0.0001$) had similar effects to that of the DEET + metal set. PB alone was not significantly different from the media control ($t = 0.15$, $df = 3$, $p = 0.89$), but PB + DU had a significantly higher production of NO compared to PB alone ($* p < 0.0001$) and PB + Sr appears to have a protective effect compared to PB alone ($* p < 0.0001$).

The PM or PM + metals exposure experiment set (Figure 2D, $F_{(9,30)} = 137.1$, $p < 0.0001$) also had similar effects to that of the other two organic + metal sets. PM alone was not significantly different from the media control ($t = 2.48$, $df = 3$, $p = 0.09$), but PB + DU had a significantly higher production of NO compared to PB alone ($* p < 0.0001$) and PB + Sr appears to have a protective effect compared to PB alone ($* p < 0.0001$).

3.3. Thiobarbituric Acid-Reactive Substances (TBARS)

Thiobarbituric acid-reactive substances (TBARS) are formed as a byproduct of lipid peroxidation, and an increase is indicative of cell oxidative stress/damage. BMEC cell cultures were exposed to a media-only control, organic compounds and metals singly, or in sets of each organic alone and in combination with each metal. Within each experiment, TBARS signal was measured and normalized to the media-only control value and presented as percent of control, where 100% represents no change from control and indicated as a horizontal dashed line in graphs. Values above this line indicate an increase in TBARS concentration, and thus a status of more stressed or damaged cell culture. In the single-compound experiments, no metal or organic compound was significantly different in a one-sample t -test of each % of control value is compared back to a value of 100 (Figure 3A). Similarly, there were no significant differences in any of the organic + metal compound groups, either between the organic compound alone compared to control, or any of the organic compound + metal groups compared back to the corresponding compound only exposure (Figure 3B) DEET + metals, ANOVA $F_{(9,30)} = 1.03$, $p = 0.44$; (Figure 3C) PB + metals, ANOVA $F_{(9,30)} = 0.55$, $p = 0.82$; (Figure 3D) PM + metals, ANOVA $F_{(9,30)} = 2.0176$, $p = 0.05$.

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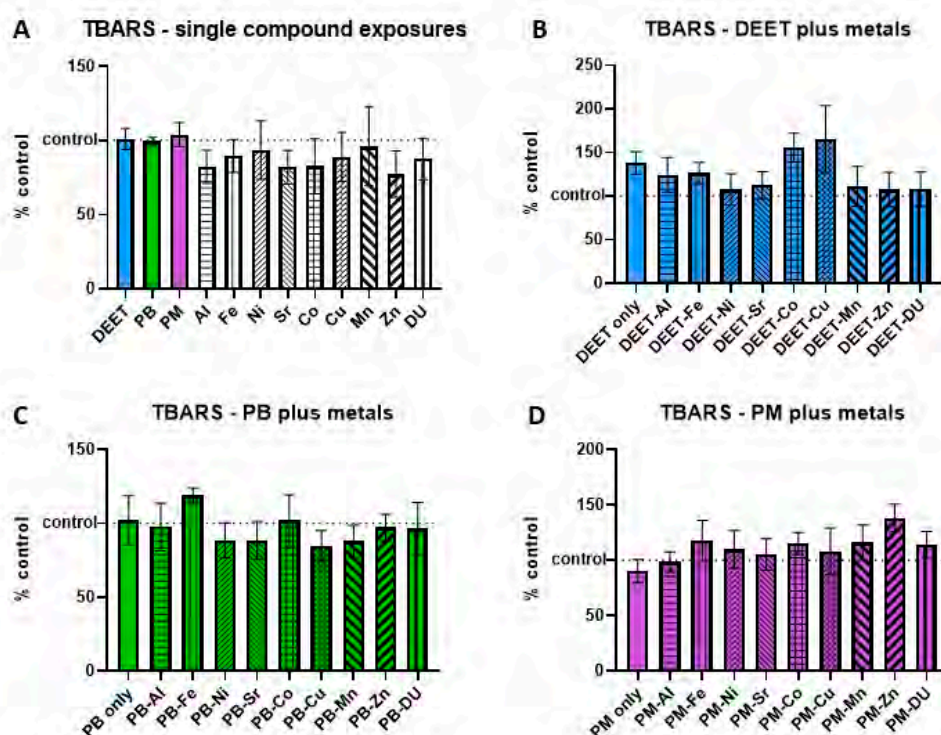


Figure 3. Thiobarbituric acid-reactive substances (TBARS) in brain microvascular endothelial cell (BMEC) cultures after organic compound and metal exposure. Assessment of thiobarbituric acid-reactive substances (TBARS) levels in BMEC cultures after exposure to organic compounds and/or metals. (A) organic compounds (10 μ M) or metals alone (1 μ M), (B) DEET (10 μ M) plus metals (1 μ M), (C) pyridostigmine bromide (PB) (10 μ M) plus metals (1 μ M), and (D) permethrin (PM) (10 μ M) plus metals (1 μ M). Dotted line at 100 indicates 100% of control measurement; values above the line indicate an increase in TBARS in the culture. Data represent the mean and standard deviation of three

(BMEC) cultures after organic compound and metal exposure. Assessment of thiobarbituric acid-reactive substances (TBARS) levels in BMEC cultures after exposure to organic compounds and/or metals. (A) organic compounds (10 μ M) or metals alone (1 μ M), (B) DEET (10 μ M) plus metals (1 μ M), (C) pyridostigmine bromide (PB) (10 μ M) plus metals (1 μ M), and (D) permethrin (PM) (10 μ M) plus metals (1 μ M). Dotted line at 100 indicates 100% of control measurement; values above the line indicate an increase in TBARS in the culture. Data represent the mean and standard deviation of three independent experiments.

3.4. Protein Carbonyl

Protein carbonyl groups are carbonyl groups that form on protein side chains upon oxidation. BMEC cell cultures were exposed to a media-only control, organic compounds and metals singly, or in sets of each organic alone and in combination with each metal. Within each experiment, protein carbonyl signal was measured and normalized to the media-only control value and presented as percent of control, where 100% represents no change from control and indicated as a horizontal dashed line in graphs. Values above this line indicate an increase in protein carbonyl concentration, and thus a status of more stressed or damaged cell culture. In the single-compound experiments, only one compound was significantly different in a one-sample *t*-test of each % of control value when compared back to a value of 100; DEET had a lower than control amount of protein carbonyl (Figure 4A, $t = 6.42$, $df = 2$, $*p = 0.02$), suggesting the possibility of a protective effect. However, this effect was not seen in the DEET + metals exposure experiment, as DEET alone compared to control media was not significant ($t = 0.09$, $df = 3$, $p = 0.94$). There were no significant differences in any of the metals in conjunction with DEET compared to DEET alone, either (Figure 4B, ANOVA $F_{(9,30)} = 0.43$, $p = 0.91$). No significant effects of PB in conjunction with metals (Figure 4C, ANOVA $F_{(9,30)} = 1.48$, $p = 0.20$) or of PM in conjunction with metals (Figure 4D, ANOVA $F_{(9,30)} = 1.81$, $p = 0.11$) were detected either.

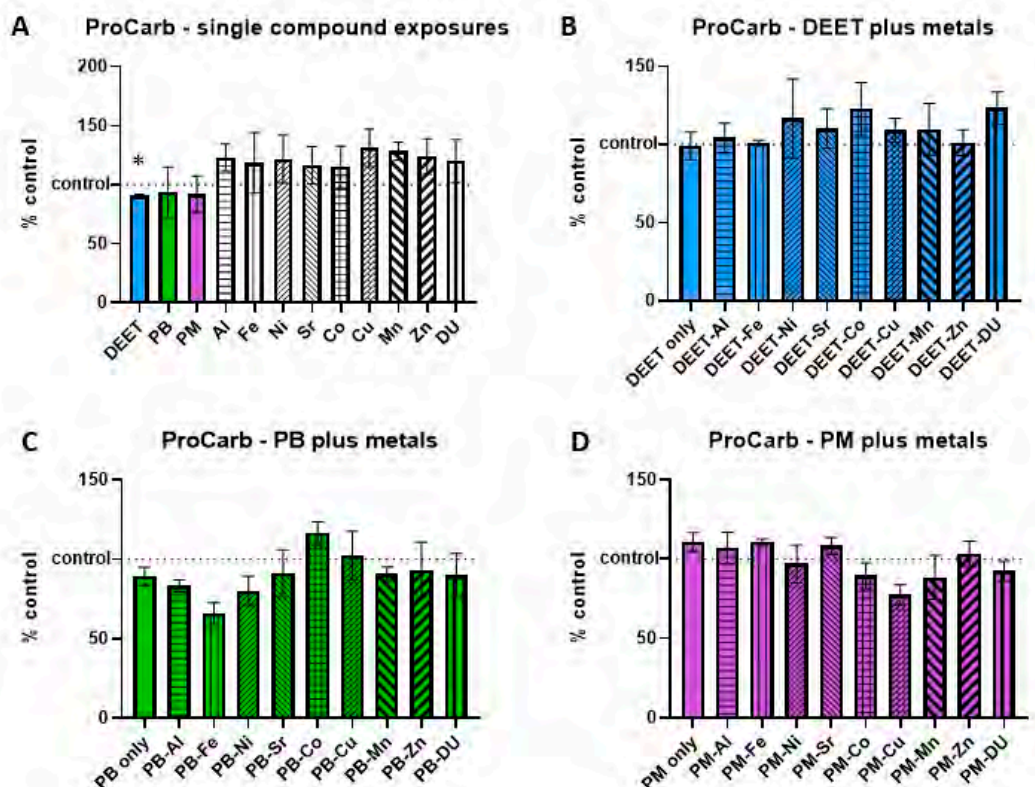


Figure 4. Protein carbonyl (ProCarb) in brain microvascular endothelial cell (BMEC) cultures after organic compound and metal exposure. Assessment of protein carbonyl levels in BMEC cultures after exposure to organic compounds and/or metals. (A) organic compounds (10 μ M) or metals alone (1 μ M), (B) DEET (10 μ M) plus metals (1 μ M), (C) pyridostigmine bromide (PB) (10 μ M) plus metals (1 μ M), and (D) permethrin (PM) (10 μ M) plus metals (1 μ M). Dotted line at 100 indicates 100% of control measurement; values above the line indicate an increase in protein carbonyl in the culture. Data represent the mean and standard deviation of three independent experiments. An * denotes a statistically significant difference from control at $p < 0.05$.

organic compound and metal exposure. Assessment of protein carbonyl levels in BMEC cultures after exposure to organic compounds and/or metals. (A) organic compounds (10 μ M) or metals alone (1 μ M), (B) DEET (10 μ M) plus metals (1 μ M), (C) pyridostigmine bromide (PB) (10 μ M) plus metals (1 μ M), and (D) permethrin (PM) (10 μ M) plus metals (1 μ M). Dotted line at 100 indicates 100% of control measurement; values above the line indicate an increase in protein carbonyl in the culture. Data represent the mean and standard deviation of three independent experiments. An * denotes a statistically significant difference from control at $p < 0.05$.

3.5. Oxidative Stress Proteins

We examined the effects of organic and metal compound exposure in BMEC cell cultures on expression oxidative stress markers (GPX4, catalase, SOD, MMP-3, and MMP-9). In each experimental set, BMECs were cultured alone and exposed to a media control, an organic compound alone, or the organic compound in combination with one of the metals. Graphs are presented as arbitrary units, where the chemiluminescence expression level of the target protein is normalized to the expression level of β -actin within each sample.

For the DEET + metals experimental set, there were no significant differences in expression of any protein after any treatment. All protein results are shown in Figure 5 (A) GPX4, $F_{(10,33)} = 0.17, p = 1.00$; (B) catalase, $F_{(10,33)} = 0.24, p = 0.99$; (C) SOD, $F_{(10,33)} = 0.34, p = 0.96$; (D) MMP-3, $F_{(10,31)} = 0.48, p = 0.89$; (E) MMP-9, $F_{(10,32)} = 0.54, p = 0.81$.

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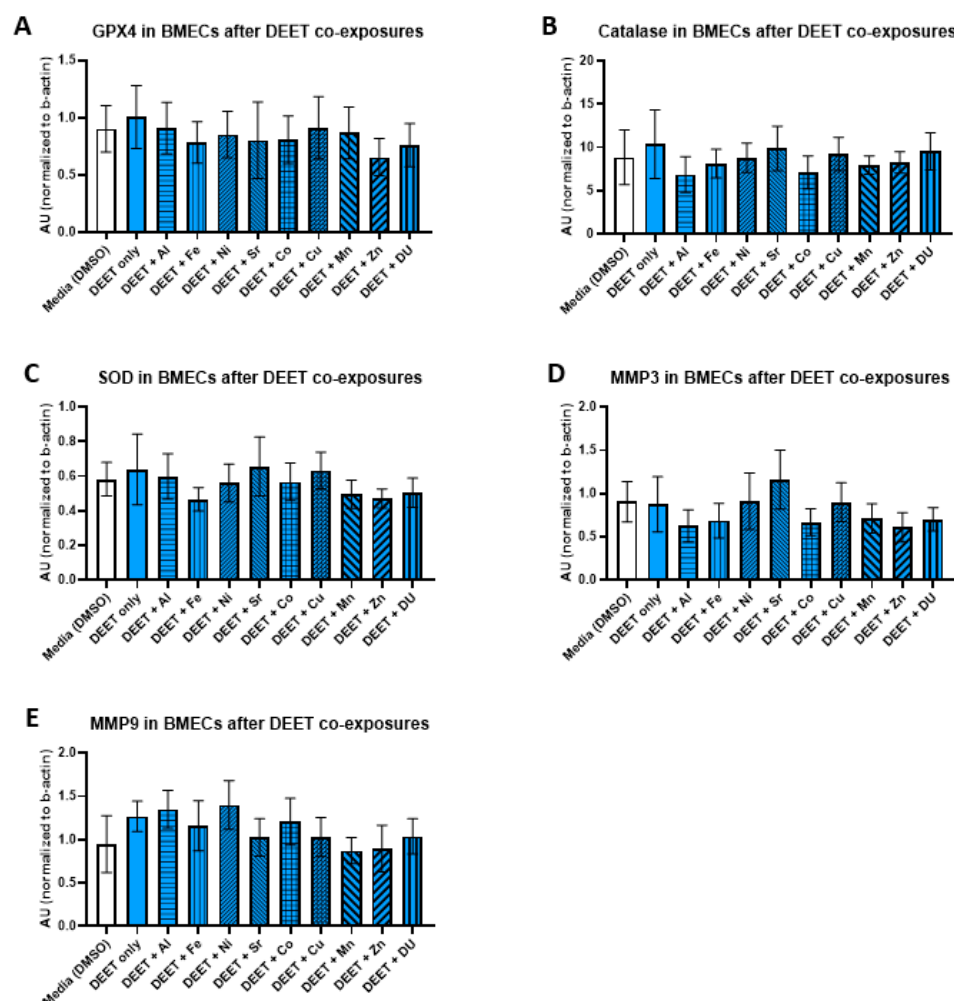


Figure 5. Oxidative stress proteins in brain microvascular endothelial cell (BMEC) cultures after DEET and metal exposure. Assessment of several oxidative stress related protein levels in BMEC cultures after exposure to DEET (10 μ M) and metals (1 μ M). (A) GPX4 expression, (B) catalase expression, (C) SOD expression, (D) MMP3 expression, and (E) MMP9 expression. All assessments are from the same experiment setup—each protein run is completed from the same homogenized sample cell pellets. DEET alone is compared to media alone, and then each DEET + metal group is compared to DEET alone. Data represent the mean and standard deviation of three independent experiments.

and metal exposure. Assessment of several oxidative stress related protein levels in BMEC cultures after exposure to DEET (10 μ M) and metals (1 μ M). (A) GPX4 expression, (B) catalase expression, (C) SOD expression, (D) MMP3 expression, and (E) MMP9 expression. All assessments are from the same experiment setup—each protein run is completed from the same homogenized sample cell pellets. DEET alone is compared to media alone, and then each DEET + metal group is compared to DEET alone. Data represent the mean and standard deviation of three independent experiments.

For the PB + metals experimental set, there were no significant differences in expression of any protein after any treatment. All protein results are shown in Figure 6 (A) GPX4, $F_{(10,33)} = 0.65$, $p = 0.76$; (B) catalase, $F_{(10,33)} = 0.24$, $p = 0.99$; (C) SOD, $F_{(10,33)} = 0.70$, $p = 0.72$; (D) MMP-3, $F_{(10,32)} = 0.36$, $p = 0.95$; (E) MMP-9, $F_{(10,32)} = 1.34$, $p = 0.25$.

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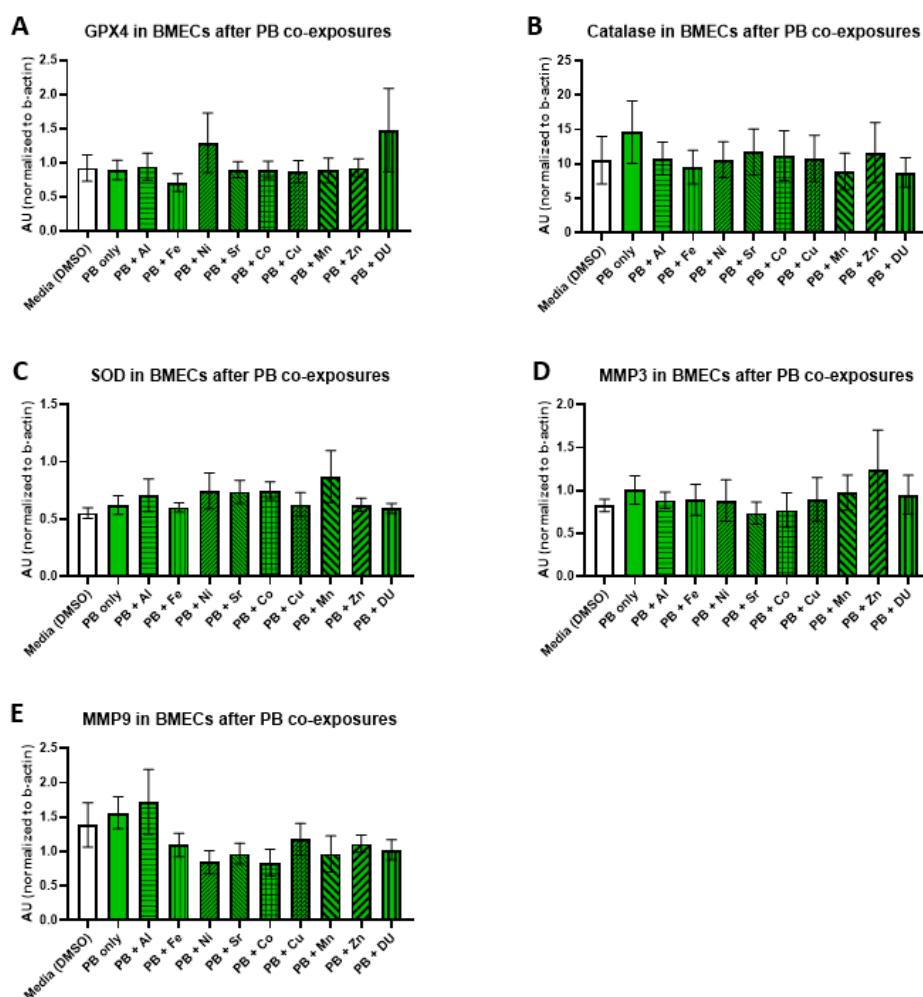
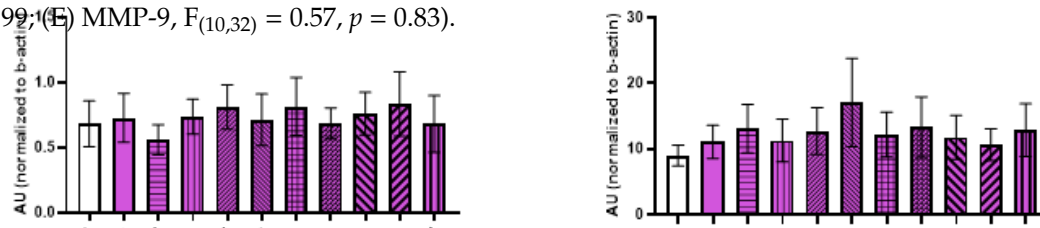


Figure 6. Oxidative stress proteins in brain microvascular endothelial cell (BMEC) cultures after pyridostigmine bromide (PB) and metal exposure. Assessment of several oxidative stress related protein levels in BMEC cultures after exposure to PB (10 μ M) and metals (1 μ M). (A) GPX4 expression, (B) catalase expression, (C) SOD expression, (D) MMP3 expression, and (E) MMP9 expression. All assessments are from the same experiment setup—each protein run is completed from the same homogenized sample cell pellets. PB alone is compared to media alone, and then each PB + metal group is compared to PB alone. Data represent the mean and standard deviation of three independent experiments.

For the PM + metals experimental set, there were also no significant differences in expression of any protein after any treatment. All protein results are shown in Figure 7 (A) GPX4, $F_{(10,32)} = 0.16$, $p = 1.0$; (B) catalase, $F_{(10,33)} = 0.99$, $p = 0.99$; (C) SOD, $F_{(10,32)} = 0.48$, $p = 0.89$; (D) MMP-3, $F_{(10,33)} = 0.20$, $p = 0.99$; (E) MMP-9, $F_{(10,32)} = 0.57$, $p = 0.83$).



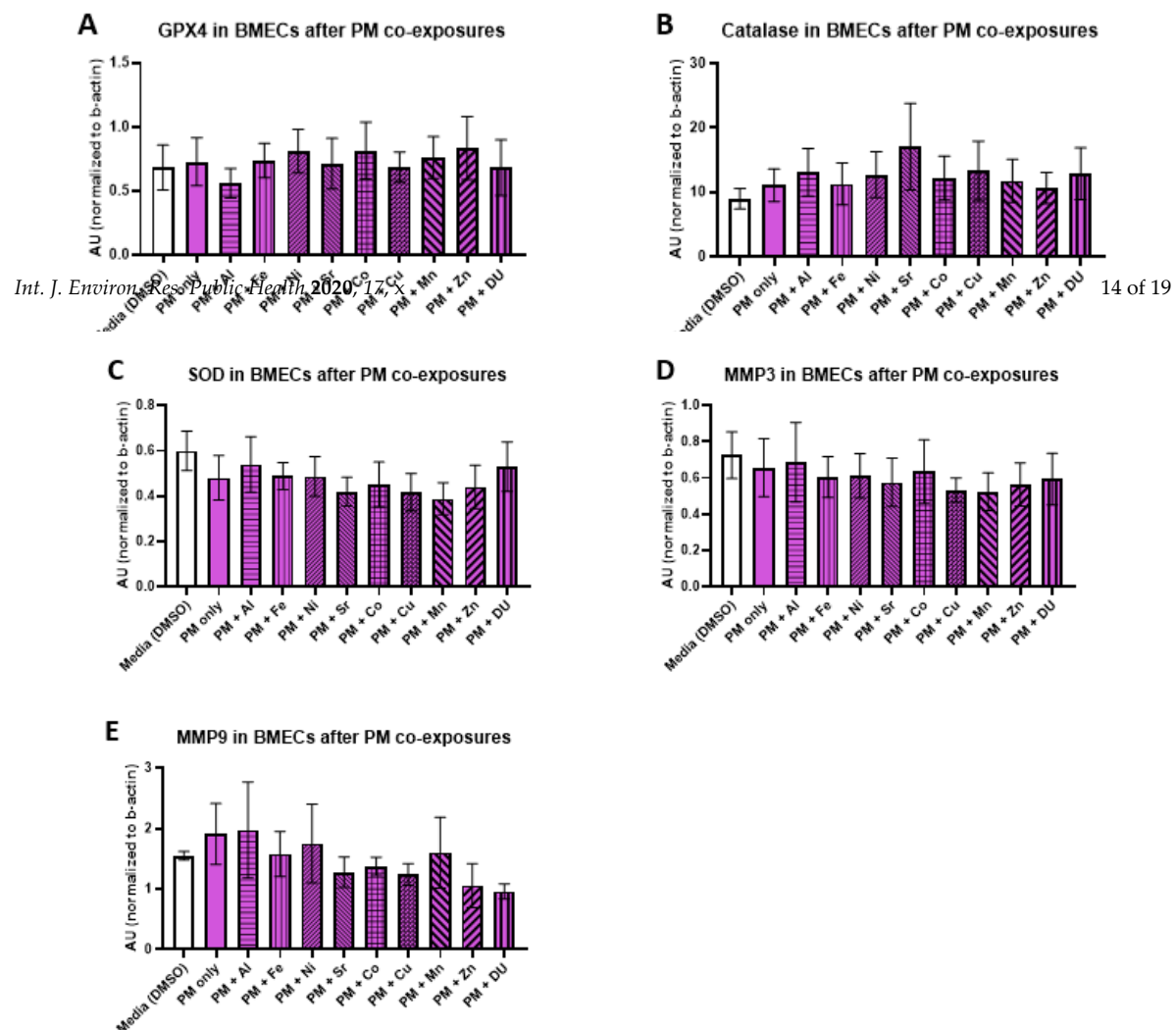


Figure 7. Oxidative stress proteins in brain microvascular endothelial cell (BMEC) cultures after permethrin (PM) and metal exposure. Assessment of several oxidative stress related protein levels in BMEC cultures after exposure to PM (10 μ M) and metals (1 μ M). (A) GPX4 expression, (B) catalase expression, (C) SOD expression, (D) MMP3 expression, and (E) MMP9 expression. All assessments are from the same experiment setup—each protein run is completed from the same homogenized sample cell pellets. PM alone is compared to media alone, and then each PM + metal group is compared to PM sample cell pellets. PM alone is compared to media alone, and then each PM + metal group is compared to PM alone. Data represent the mean and standard deviation of three independent experiments.

4. Discussion

Given the multiple symptom profile associated with Gulf War Illness and that so many military personnel experiencing GWI do not all share known single-exposure scenarios, it is likely that multiple factors are at work. One proposed multi-exposure scenario is a combination of exposure to organic compounds in the field at the time, such as N,N-diethyl-*m*-toluamide (DEET), pyridostigmine bromide (PB), or permethrin (PM), and metals found in the “desert dust” that was inhaled and internalized. The blood-brain barrier serves to heavily restrict movement of molecules between the blood and central nervous system (CNS) [50–52]. Most of the properties of the BBB are due to endothelial cells and supporting astrocyte cells, and if this were to be disrupted and allow the passage of compounds such as metals or viral particles into the CNS, it could contribute to the fatigue, anxiety, depression, and other cognitive problems seen in GWI. We hypothesized that exposures of sub-toxic levels of DEET, PB, and PM, particularly in combination with concurrent exposure to sub-toxic levels of metals, would adversely affect the health and viability of cells involved in the BBB, which could lead to subtle changes in permeability and function. In running a dose range of organic compounds on BMECs and astrocytes, we found that in general there was a tipping point between 10 μ M and 100 μ M in toxicity of both cell types, although this change was much stronger in PM exposure than DEET or PB (Supplementary Materials Figure S2). Similarly, many metal treatments had a tipping point

changes in permeability and function. In running a dose range of organic compounds on BMECs and astrocytes, we found that in general there was a tipping point between 10 μ M and 100 μ M in toxicity of both cell types, although this change was much stronger in PM exposure than DEET or PB (Supplementary Materials Figure S2). Similarly, many metal treatments had a tipping point after the 10 μ M dose in both BMEC and astrocyte cultures (Supplementary Materials Figures S3 and S4), but enough started a downward trend after only 1 μ M that we chose that as an across-the-board sub-toxic dose for subsequent experiments. It is important to note that metals had rather different dose responses, though, and more specific follow-up at other exposure concentrations would be worth examining. For example, Sr stayed close to control toxicity levels in both BMECs and astrocytes across all doses tested, Al actually increased viability markers in BMECs over several doses, and Mn and Cu had downward trending curves from a much lower dose in BMECs and astrocytes. We also report that in BMEC cultures, Co and Mn in conjunction with each of the three organic compounds were significantly more toxic than the corresponding organic compound alone. Mn neurotoxicity has been well studied [53–55]. In fact, using RBE cells, a rat brain endothelial cell line, dos Santos and colleagues showed that Mn demonstrated significant toxicity in a dose range of 200–800 μ M and concluded that Mn induced mitochondrial injury in these cells [56]. We observed Mn toxicity at much lower concentrations, both alone as well as in combination with Gulf War-associated organics. Although beyond the scope of this study, the role of mitochondria in the potentiation of combined organic/metal exposures, especially in light of the important role the mitochondria play in the production of reactive oxygen and nitrogen species [57], would add to our understanding of these unique exposure scenarios.

In evaluating oxidative stress markers, DU appears to have the most effect on BMEC cultures. DU alone increased nitric oxide levels, and DEET plus DU exposure increased nitric oxide levels, although DEET alone had no effect. DU increased nitric oxide levels in co-exposure with both the other organic compounds, PB and PM, as well, suggesting internalized DU could be a greater risk to brain function if the person is also exposed to prophylactic or insecticide compounds at the same time. It is not clear whether the increase in nitric oxide in the co-exposure scenarios was the result of a synergistic effect of DU with the organic compound, or solely ascribed to DU. Interestingly, combinations of Sr with each of the three organic compounds reduced nitric oxide production, suggesting a protective effect; this could be tied to the relative lack of toxicity seen across the tested dose range of Sr in cell culture. Strontium has the ability to replace calcium in many systems [58]; however, it is not known whether this property can explain this finding. DEET may also have a propensity to increase protein carbonyl levels in BMECs, but this exposure dose could be on a cusp of toxicity given a significant difference was found in one experiment but not another. We did not see effects of the organic compounds or metals, alone or in combination, on TBARS levels, or oxidative stress marker protein expression of GPX4, catalase, SOD, MMP-3, or MMP-9. Although we did not observe significant effects on the cell's antioxidant defenses (GPX4, catalase, SOD) or induction of damage-associated proteins (MMP-3, MMP-9) at the doses we tested, there have been reports in the literature suggesting that metal exposure can affect these enzyme systems [59–61]. Many of the metals tested can also function as catalysts in the Fenton and Haber-Weiss reactions and are capable of generating highly reactive oxygen and nitrogen species [62–64]. It has been postulated that these reactions play a major role in metal-induced neurodegeneration [65,66].

A limitation of this study, in fact of any study using an in vitro or animal model system, is the extrapolation to the human condition. In this regard, as noted earlier, record keeping with respect to the various types of exposure, especially pyridostigmine bromide administration, in the First Gulf War were spotty at best. Thus actual in-theatre concentration levels of the various compounds encountered are not known. Experimentally, this necessitates an initial cytotoxicity assessment of the various test compounds being studied. Whether the concentrations used in these in vitro studies correlate to actual human exposure levels in the First Gulf War remain to be seen.

The effects we report here are subtle, but suggest implications on degraded function of the BBB as a result of these unique Gulf War-related exposures. In future work we will examine changes in BBB

function more closely using a BMEC and astrocyte co-culture transwell system as a more accurate 3D model of BBB vasculature, rather than traditional cell culture plates for toxicity assays.

5. Conclusions

Human BMEC and human astrocyte cell cultures were evaluated for toxicity across a range of concentrations of organic compounds (N,N-diethyl-*m*-toluamide (DEET), permethrin, and pyridostigmine bromide) and metals (aluminum, iron, nickel, strontium, cobalt, copper, manganese, zinc, and depleted uranium) that are relevant to potential exposure scenarios for US military personnel during the Gulf War. We determined a general sub-toxic dose of 10 μ M for the three organic compounds and 1 μ M for the nine metals of interest and used this to examine markers of viability and oxidative stress on BMEC cell cultures in co-exposure scenarios for the organic and metal compounds, as these would be more relevant to actual exposure scenarios for US military personnel and thus may contribute to Gulf War Illness symptoms. Many markers were not affected, or were subtle, but we found a significant increase in nitric oxide after exposure to any of the three compounds in conjunction with depleted uranium. Future work will determine how much, if any, effect this has on blood–brain barrier function.

Supplementary Materials: The following are available online at <http://www.mdpi.com/1660-4601/17/22/8336/s1>, Figure S1: Immunohistochemistry of BMEC markers; Figure S2: Toxicity of organic compounds on BMECs and astrocytes; Figure S3: Toxicity of metals on BMECs; Figure S4: Toxicity of metals on astrocytes; Figure S5a: Microscopy of BMECs exposed to a dose range of organic or metal compounds; Figure S5b: Microscopy of BMECs exposed to a dose range of organic or metal compounds; Figure S6a: Microscopy of astrocytes exposed to a dose range of organic or metal compounds; Figure S6b: Microscopy of astrocytes exposed to a dose range of organic or metal compounds; Figure S7: Example of ProteinSimple run data; Table S1: Antibody Table.

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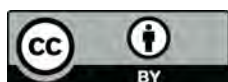
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Table S1 –Antibody Table

Details for antibodies used in fluorescent immunohistochemistry and protein expression analysis.

Figure S1 – Immunohistochemistry of BMEC markers

Microscopy images of BMEC cultures to verify expression of BBB relevant markers occludin and ZO-1 using fluorescent immunohistochemistry. Images are at 10X magnification, scale bar represents 50 μ m. A) image of BMEC culture stained with occludin in red, DAPI (for nuclei) in blue. B) image of BMEC culture stained with ZO-1 in red, DAPI in blue.

Figure S2 – Toxicity of organic compounds on BMECs and astrocytes

Dose response curves of individual treatments with organic compounds in BMEC (A-F) or astrocyte (G-L) cultures. Toxicity and viability were determined with MTT or NR assays for each cell type and organic combination. Dotted line at 100 indicates 100% of control measurement: values below the line indicate the dose is toxic to the cells and viability is decreased. Data represent the mean and standard deviation of three independent experiments.

Figure S3 – Toxicity of metals on BMECs

Dose response curves of individual treatments with metals in BMEC cultures. Toxicity and viability were determined with MTT (A-I) or NR (J-R) assays for each cell type and organic combination. Dotted line at 100 indicates 100% of control measurement: values below the line indicate the dose is toxic to the cells and viability is decreased. Data represent the mean and standard deviation of three independent experiments.

Figure S4 – Toxicity of metals on astrocytes

Dose response curves of individual treatments with metals in astrocyte cultures. Toxicity and viability were determined with MTT (A-I) or NR (J-R) assays for each cell type and organic combination. Dotted line at 100 indicates 100% of control measurement: values below the line indicate the dose is toxic to the cells and viability is decreased. Data represent the mean and standard deviation of three independent experiments.

Figure S5a – Microscopy of BMECs exposed to a dose range of organic or metal compounds

Giemsa stained BMEC cell cultures after exposure to metals or organic compounds, paired with Supplemental Figure 4b.

Figure S5b – Microscopy of BMECs exposed to a dose range of organic or metal compounds

Giemsa stained BMEC cell cultures after exposure to metals or organic compounds, paired with Supplemental Figure 4a.

Figure S6a – Microscopy of astrocytes exposed to a dose range of organic or metal compounds

Giemsa stained astrocyte cell cultures after exposure to metals or organic compounds, paired with Supplemental Figure 5b.

Figure S6b – Microscopy of astrocytes exposed to a dose range of organic or metal compounds

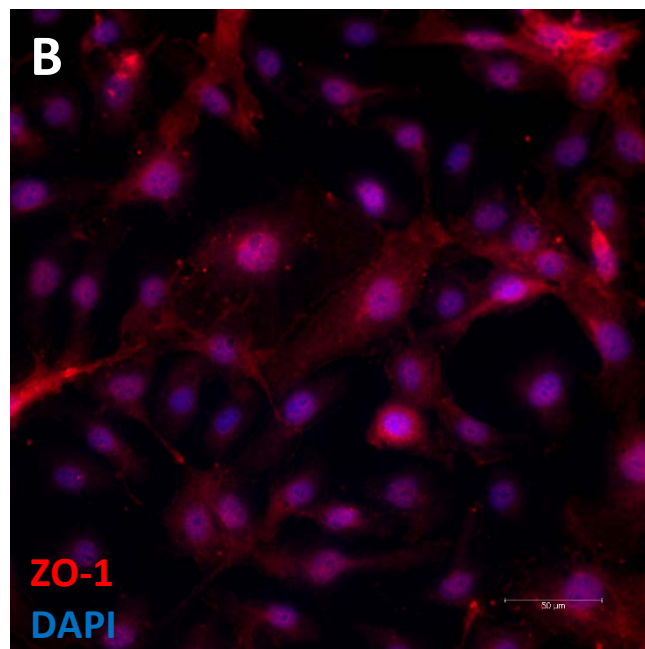
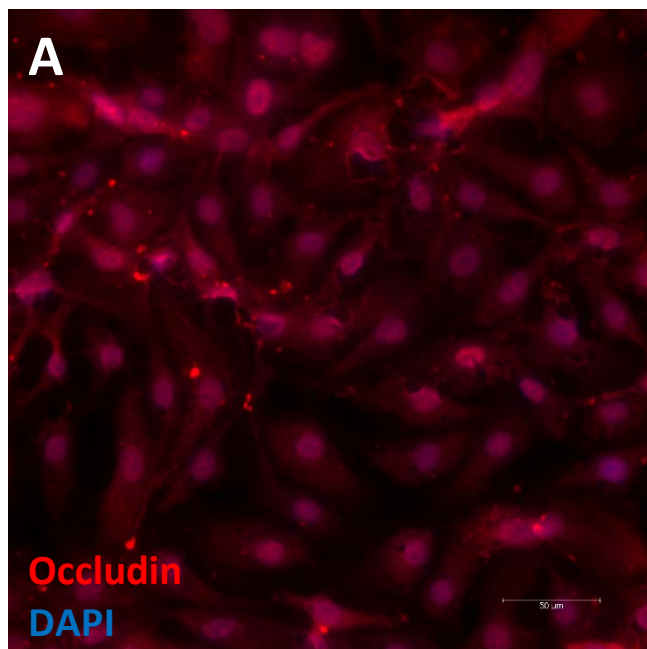
Giemsa stained astrocyte cell cultures after exposure to metals or organic compounds, paired with Supplemental Figure 5a.

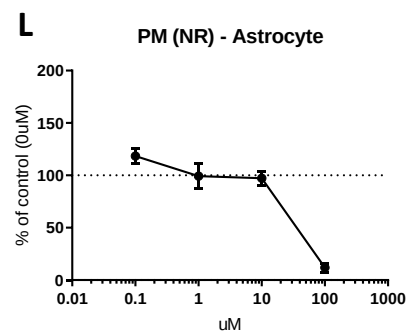
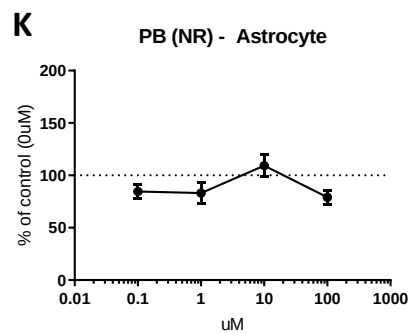
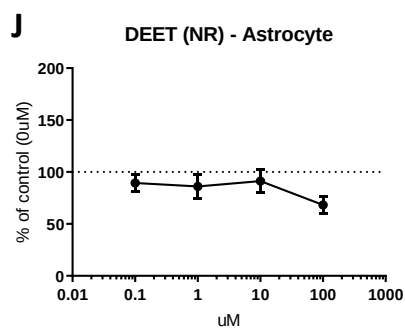
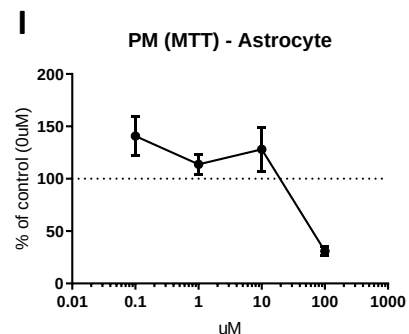
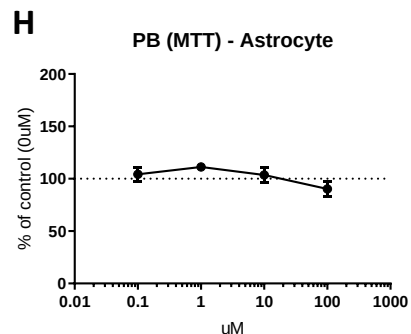
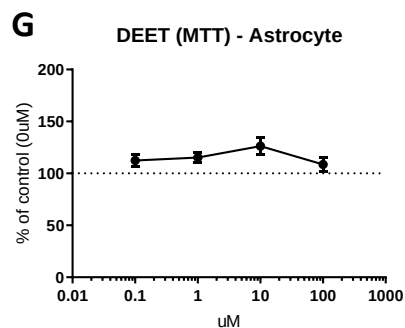
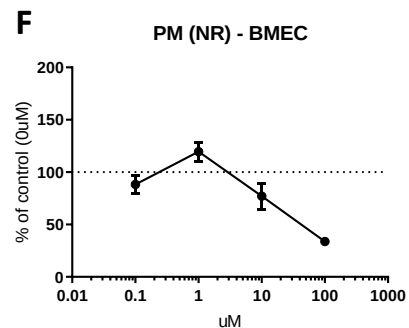
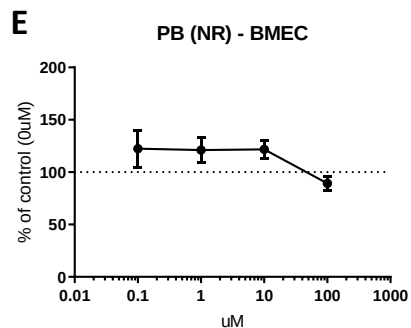
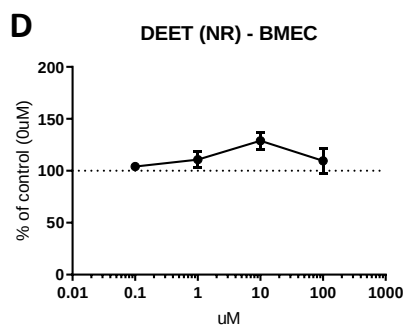
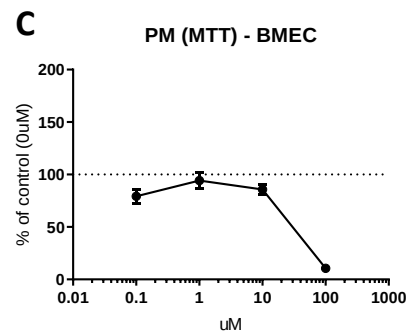
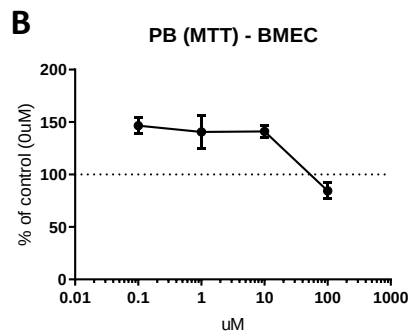
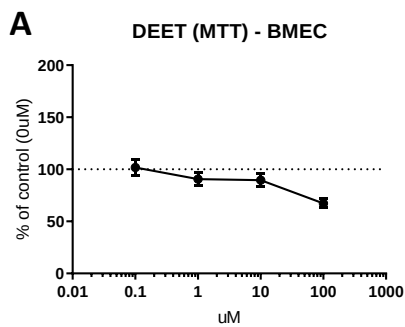
Figure S7 – Example of ProteinSimple run data

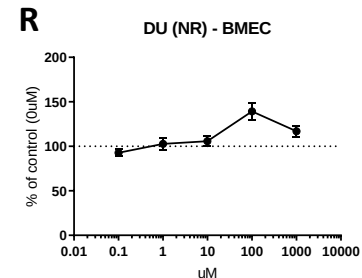
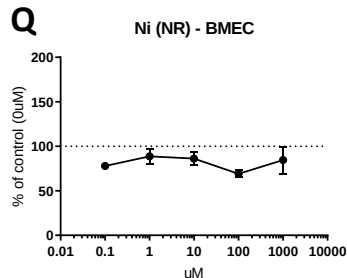
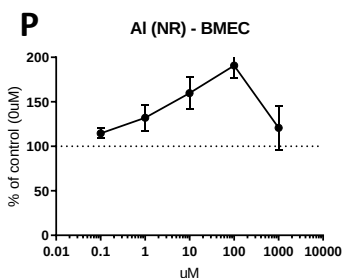
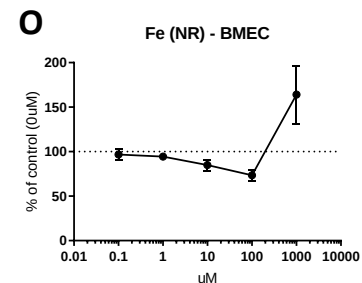
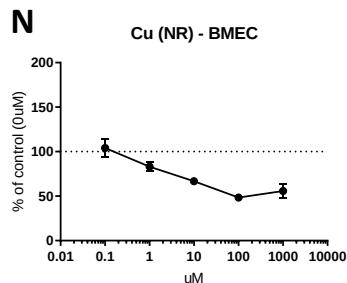
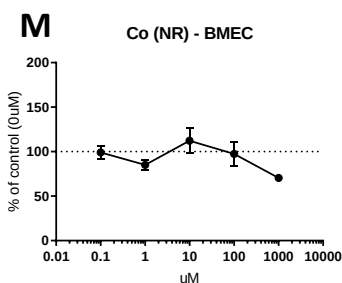
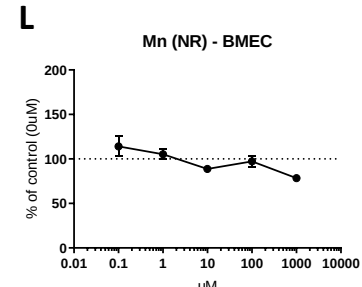
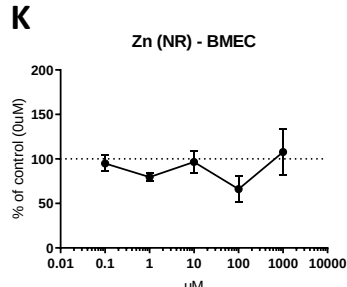
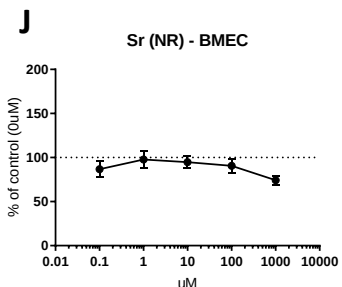
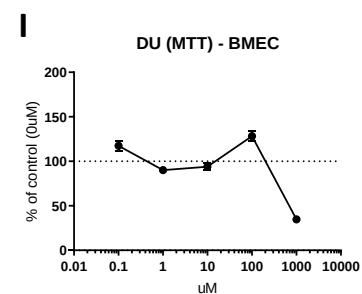
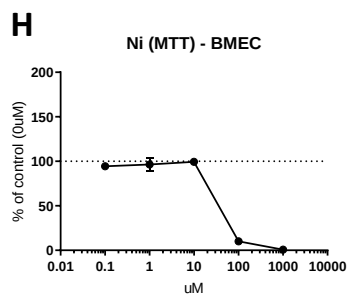
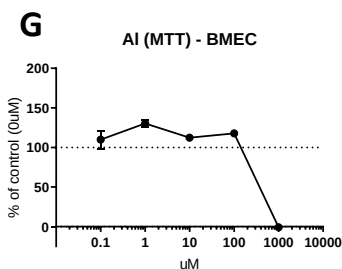
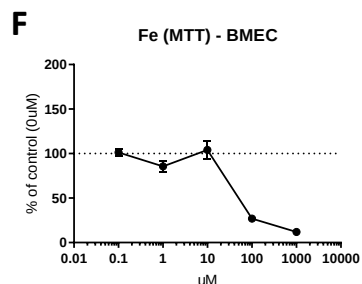
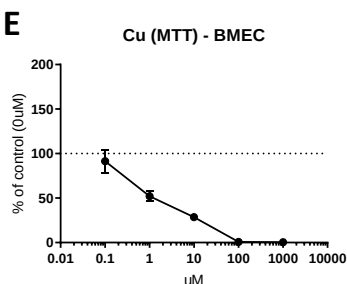
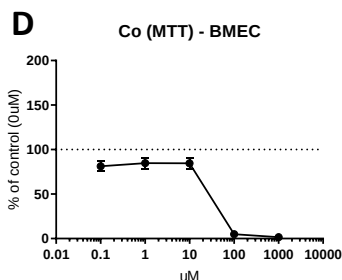
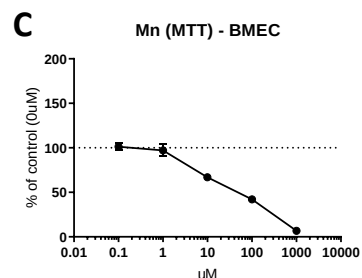
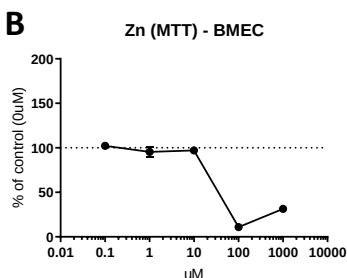
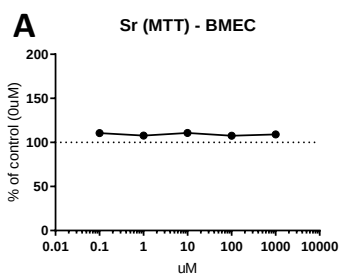
Rather than a traditional Western blot with gels and membranes for analysis of protein expression, the ProteinSimple Wes system uses a capillary-based size separation method and chemiluminescent detection. Analysis of band intensity is performed directly in the Wes machine, automatically calculating area under the peak. The Compass program software is also capable of generating an image like that of a traditional Western blot. The chemiluminescence values for a sample set of DEET+metal BMEC experiment is shown in (A), and the corresponding “Western blot” image generated from those values is shown in (B). GPX4 and β -actin are labeled in both images.

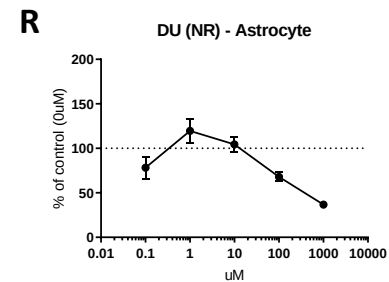
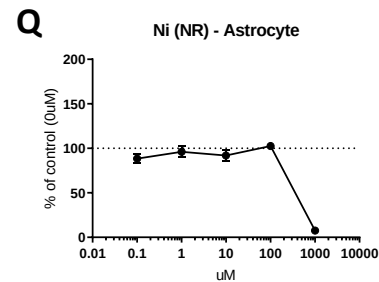
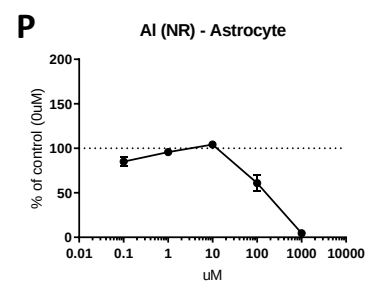
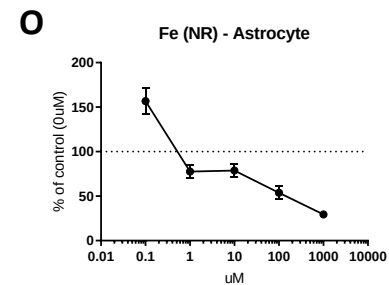
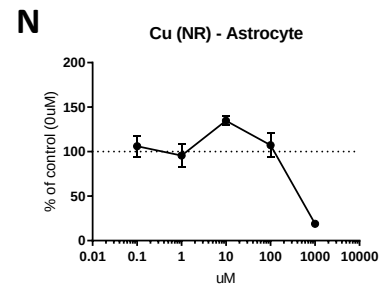
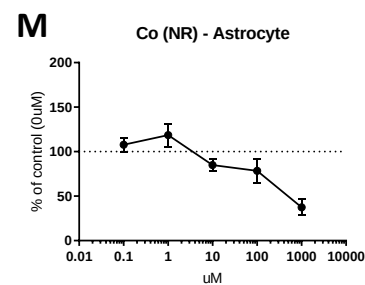
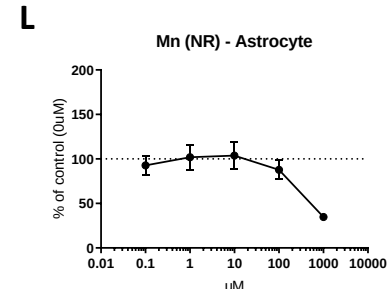
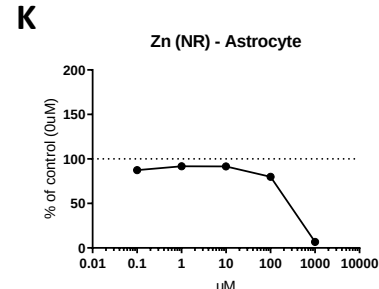
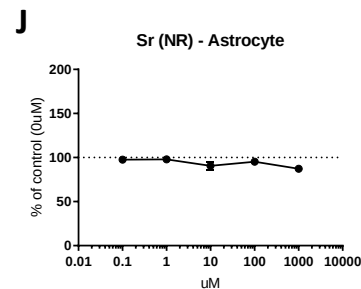
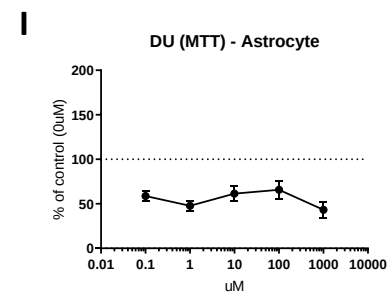
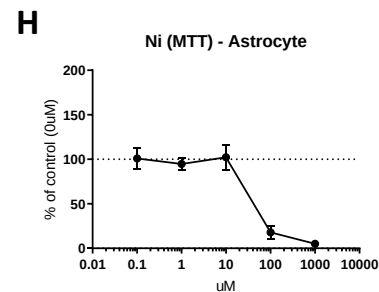
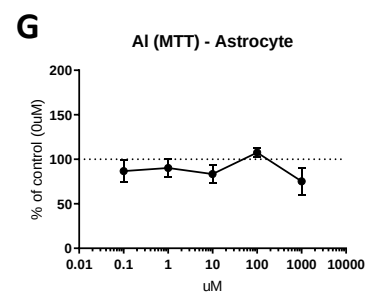
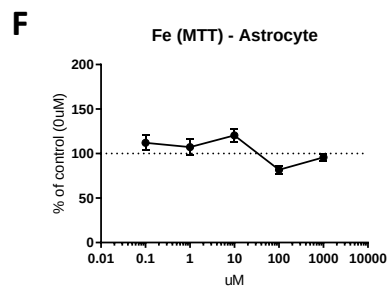
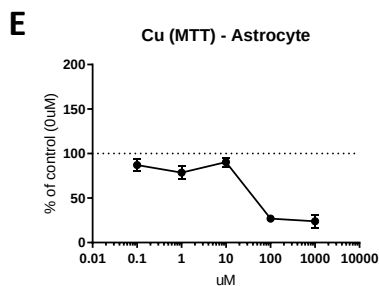
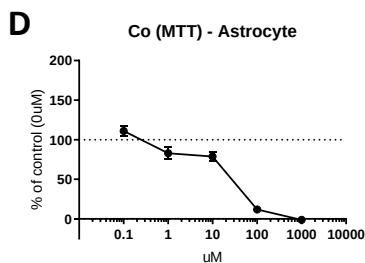
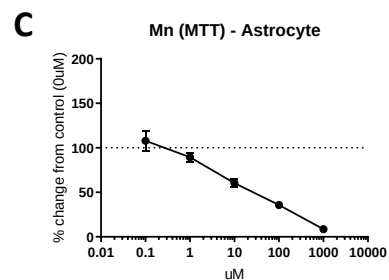
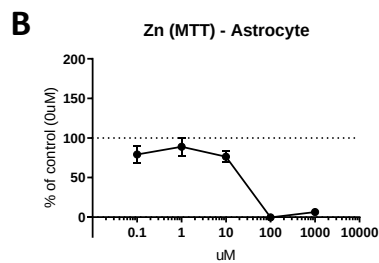
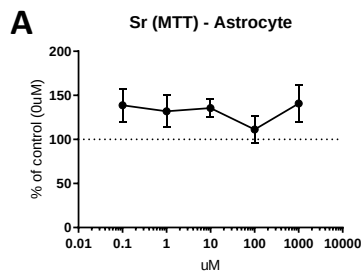
Antibody Table

Primary Antibodies					
Antibody	Host	Vendor, Cat #	Purpose	Conc.	Size (kDa)
ZO-1	Rb	ThermoFisher 40-2200	BBB marker	1:50	
Occludin (N-term)	Rb	ThermoFisher 40-6100	BBB marker	1:50	
Alexa Fluor 555 (red)	Dnk	Abcam ab150074	Fluorescent 2°	1:1000	
DAPI (blue)		ThermoFisher D1306	Nuclei stain	1 µg/ml	
Superoxide dismutase (SOD)	Rb	Abcam, Ab13498	Antioxidant defense	1:50	25
Glutathione peroxidase (GPX4)	Ms	Novusbio, MAB5457	Antioxidant defense	1:50	25
Catalase	Ms	Novusbio, MAB3398	Antioxidant defense	1:200	62
MMP3	Rb	Abcam, Ab53015	Cell damage	1:50	54
MMP9	Rb	Abcam, 38898	Cell damage	1:50	92
Beta-actin (13E5) x rab		CellSignaling, 4970S	Housekeeping (normalization)	1:10,000	48
Beta-actin (8H10D10) x mou		CellSignaling, 3700S	Housekeeping (normalization)	1:10,000	48









Endothelial Cells (10X)

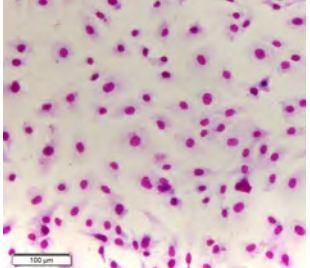
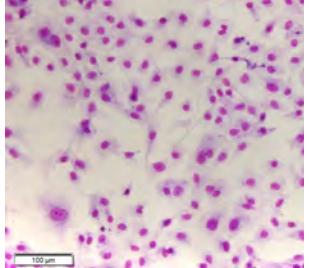
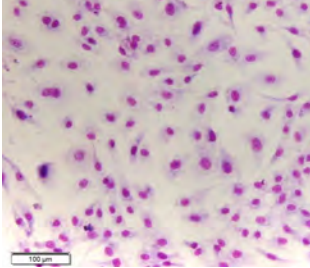
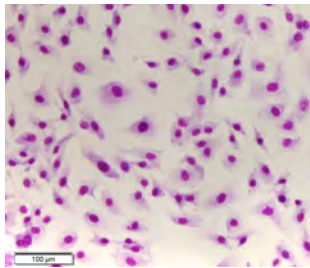
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1 μ M

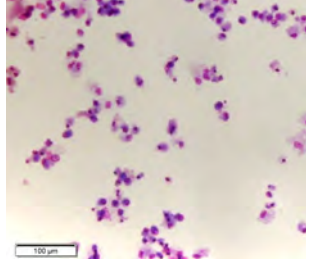
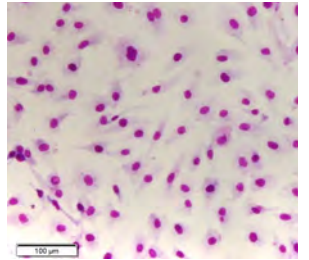
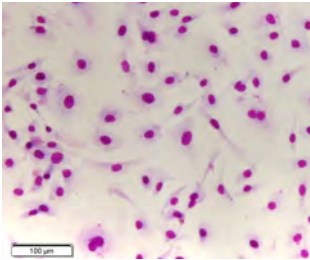
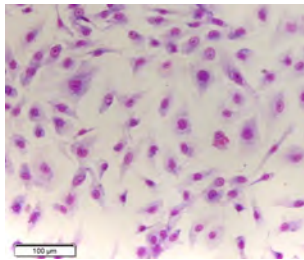
10 μ M

100 μ M

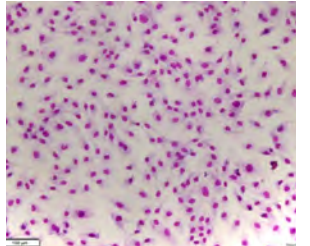
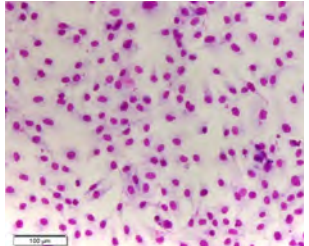
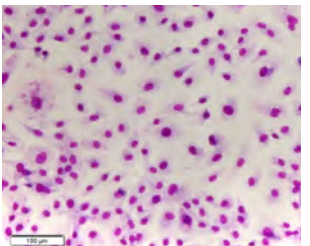
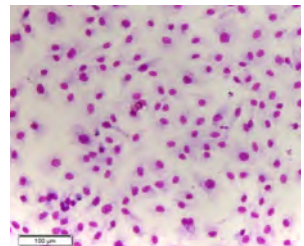
Sr



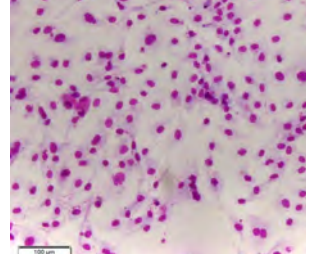
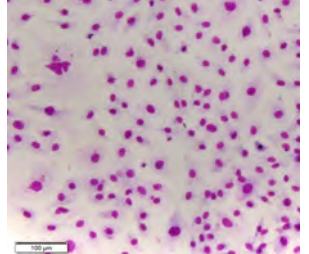
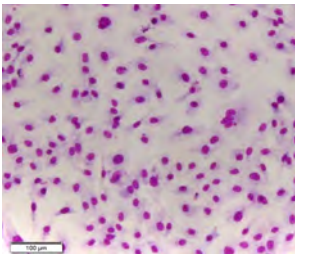
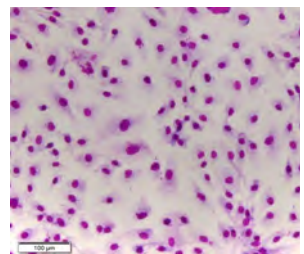
Zn



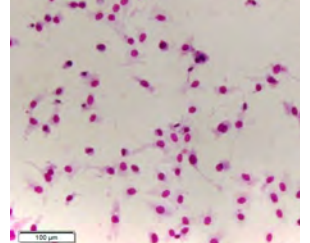
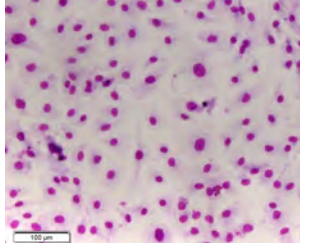
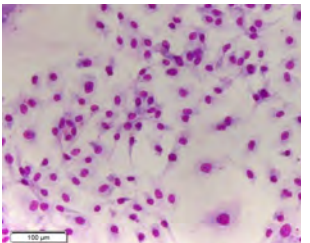
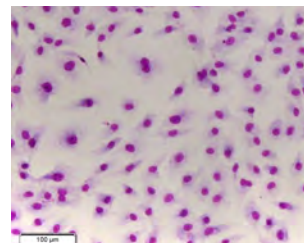
Mn



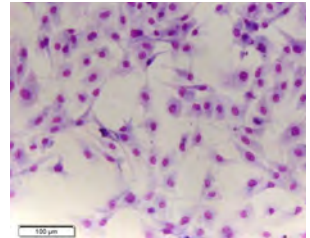
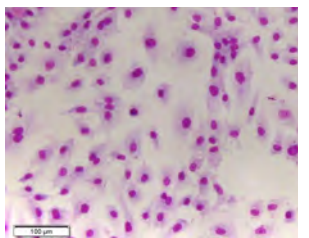
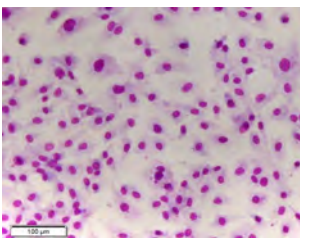
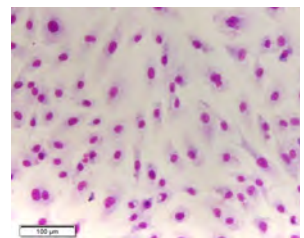
Co



Cu



Fe



Endothelial Cells (10X)

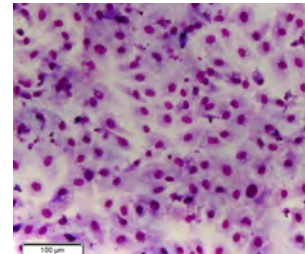
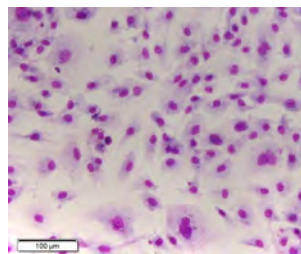
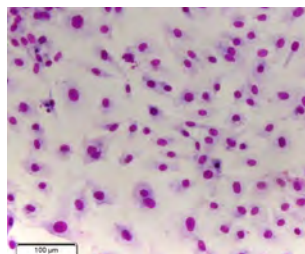
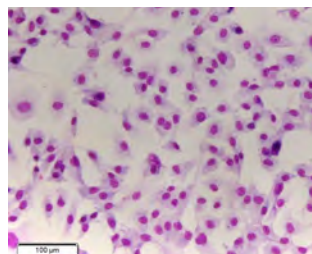
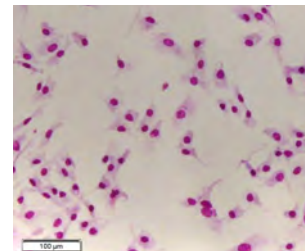
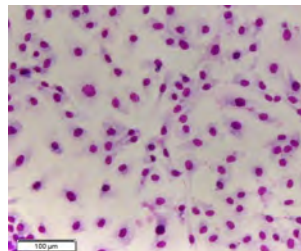
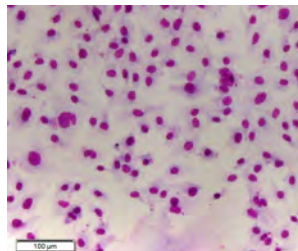
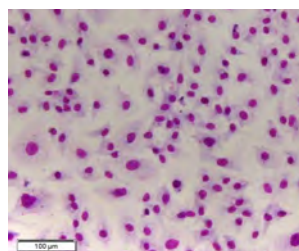
0 μ M

1 uM

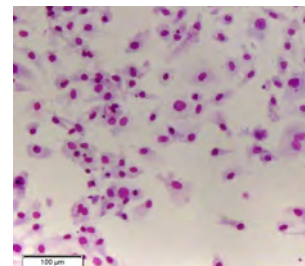
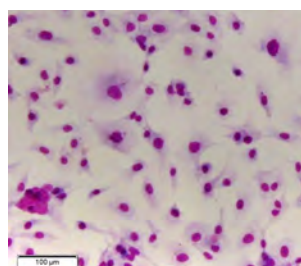
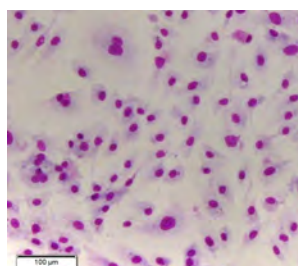
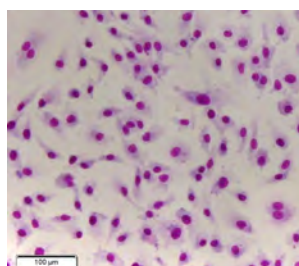
10 μ M

100 μ M

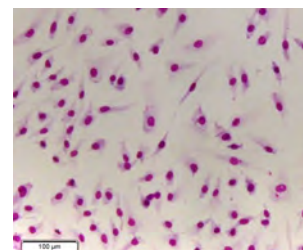
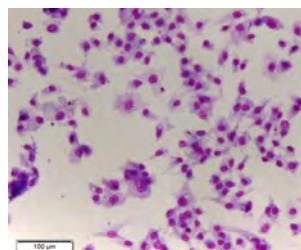
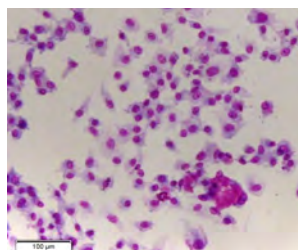
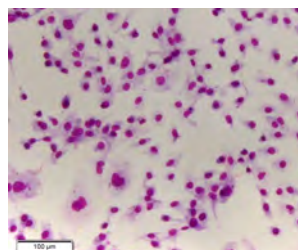
AI

 $\ddot{z}$ 

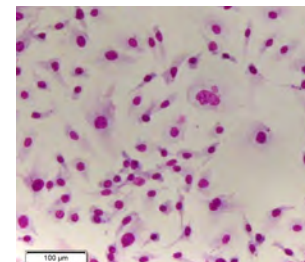
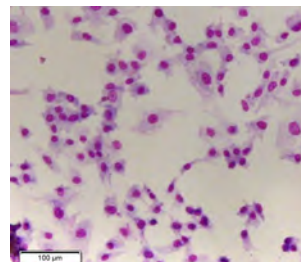
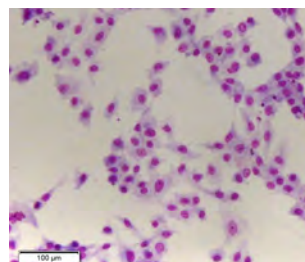
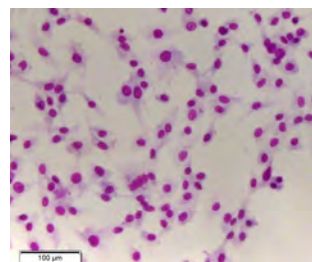
ד



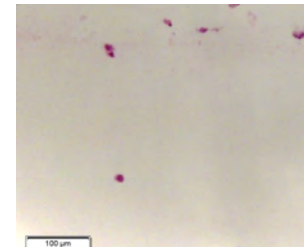
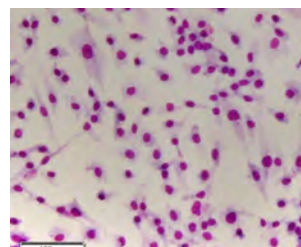
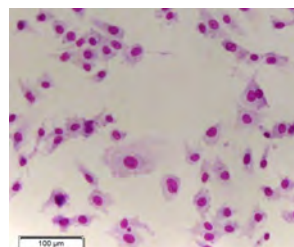
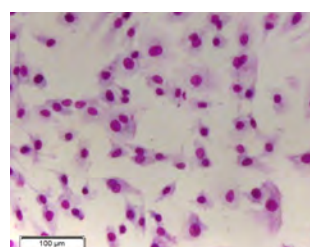
DEET



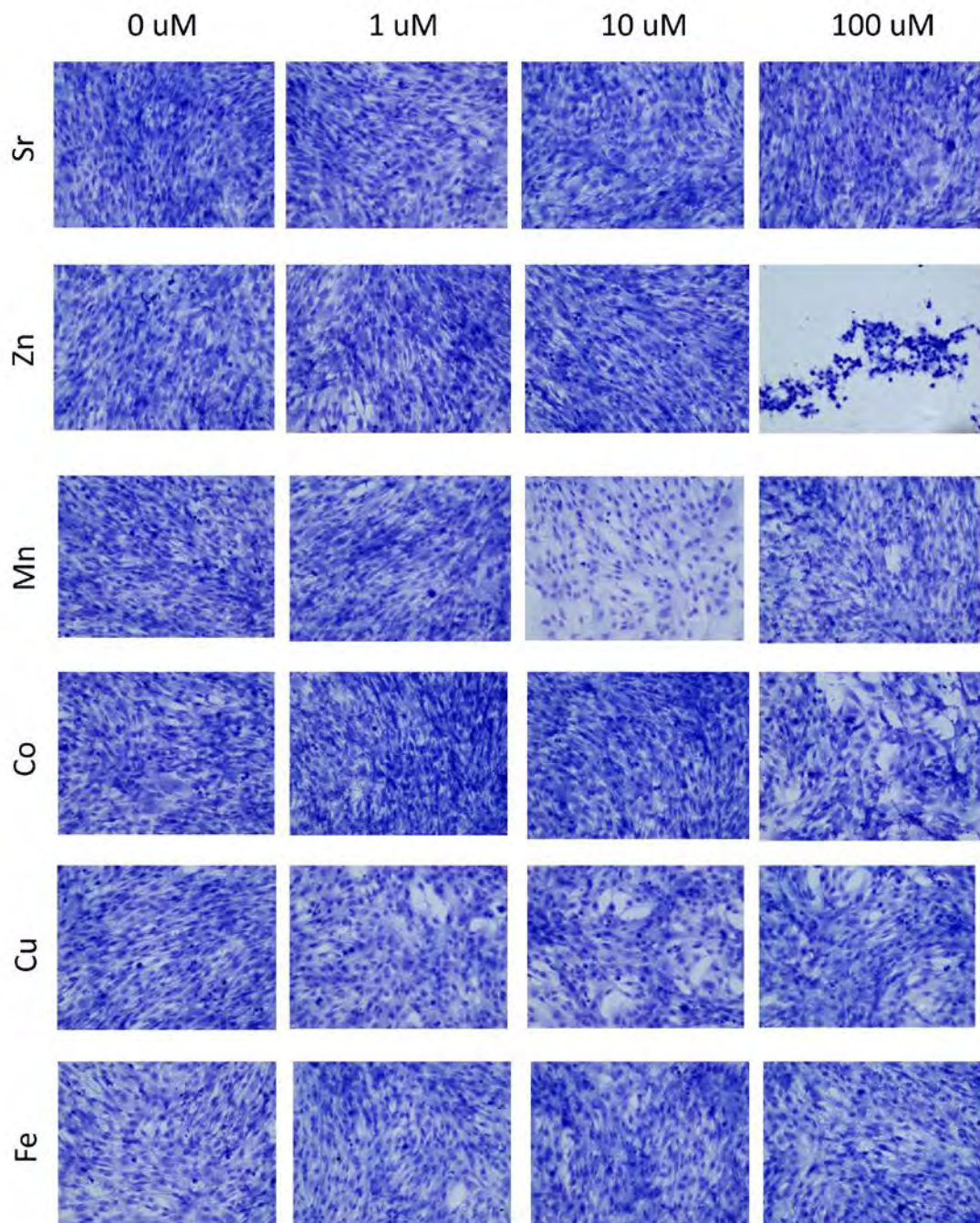
PB

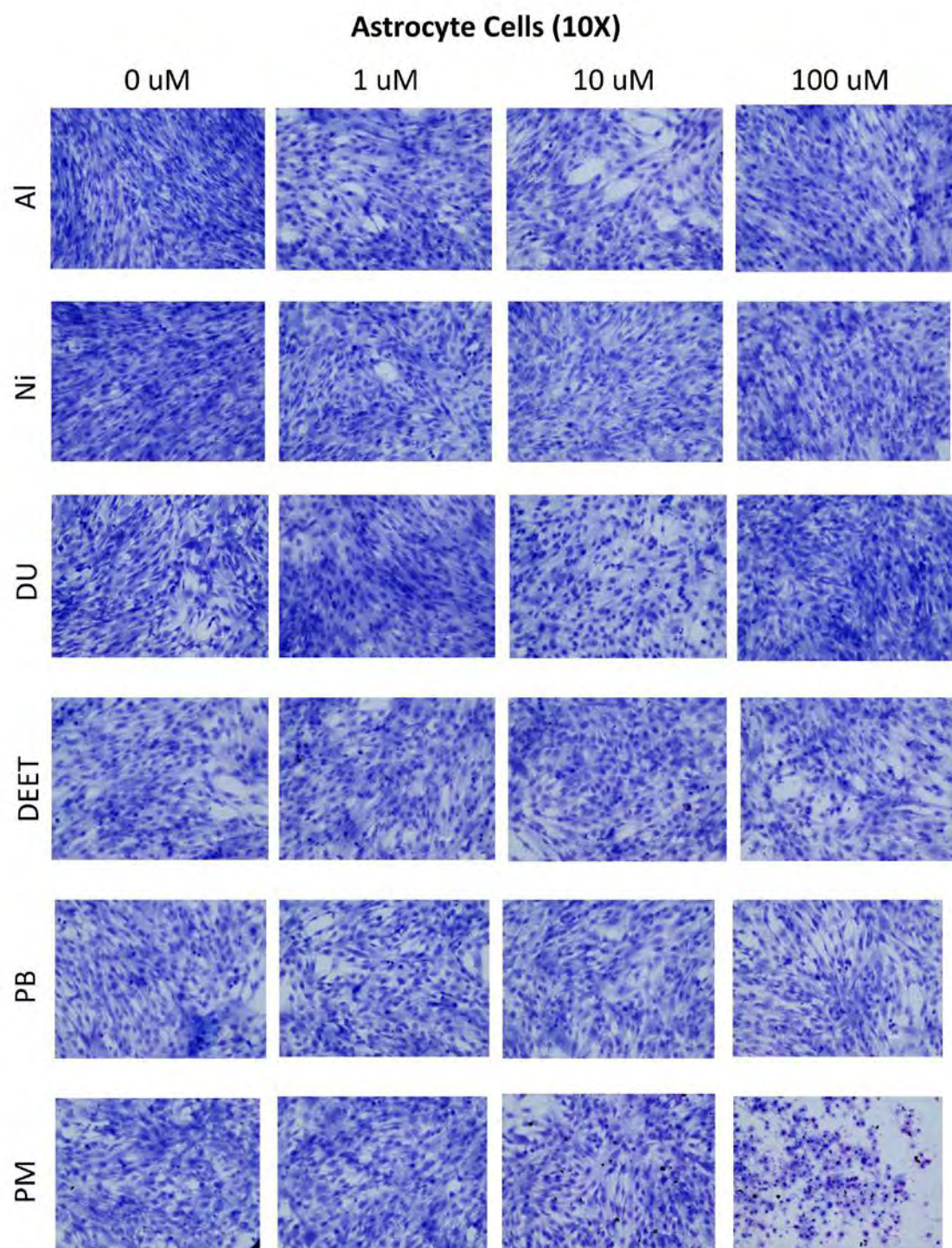


PM

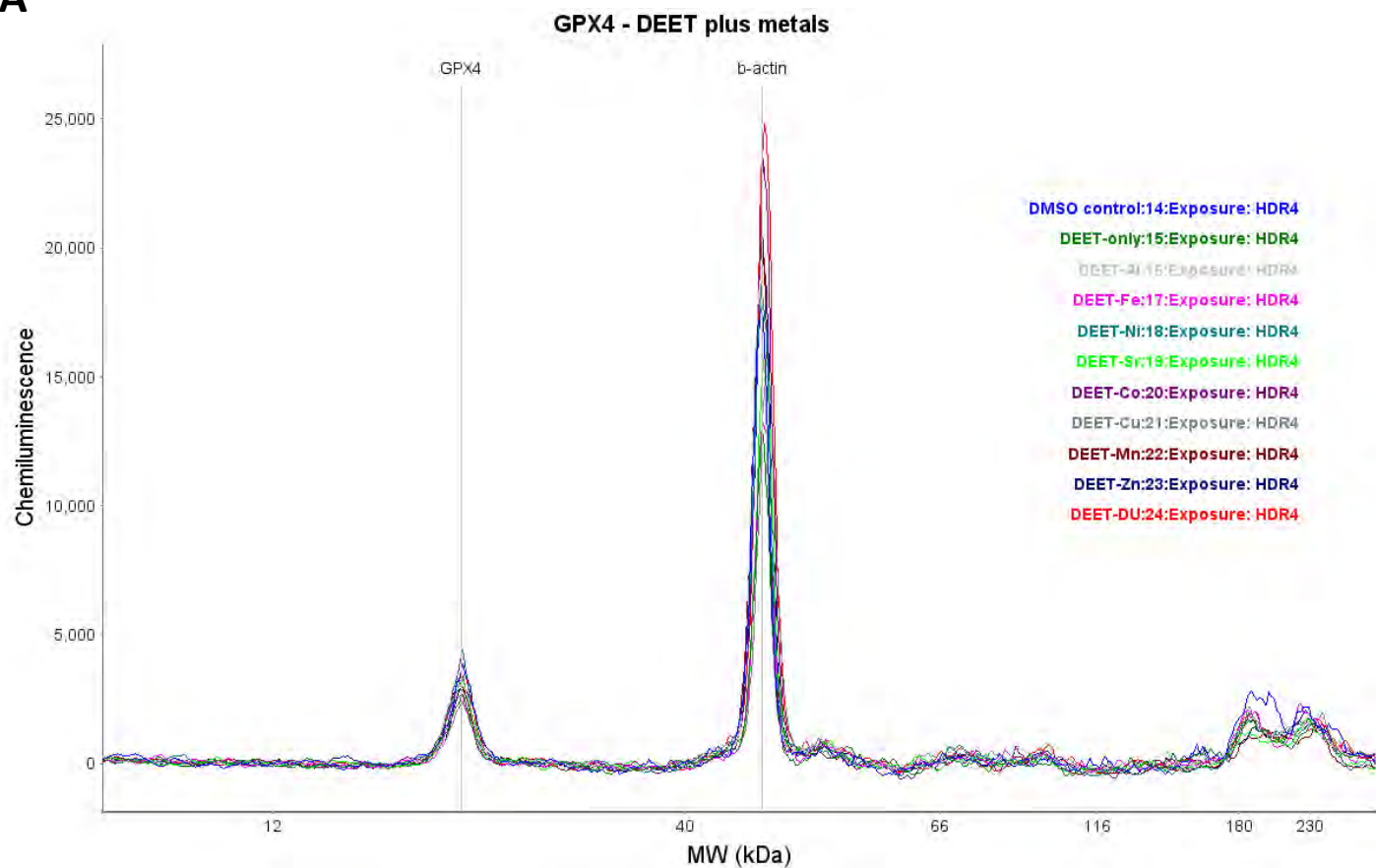


Astrocyte Cells (20X)

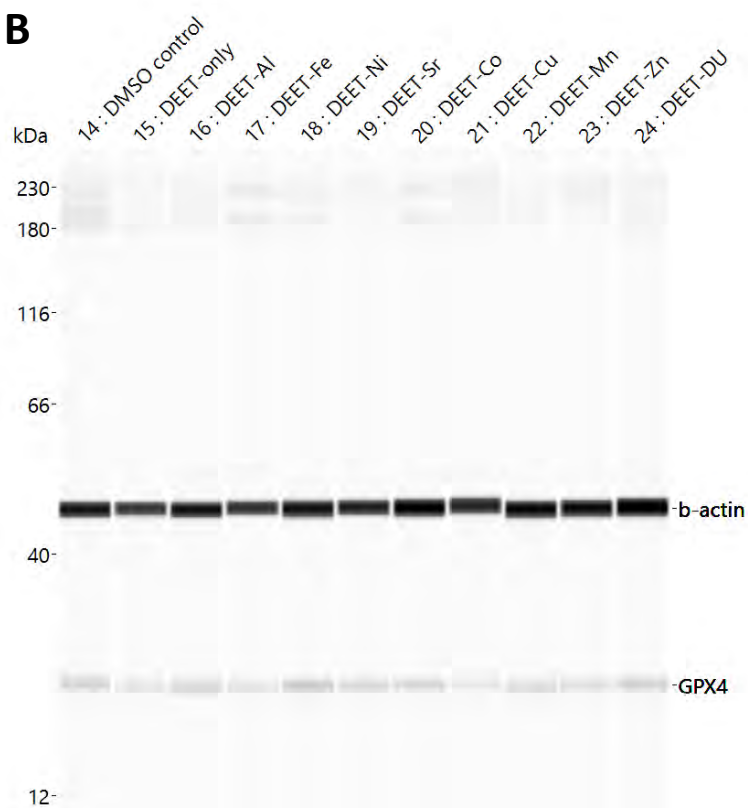




A



B



The Gulf War-associated compounds DEET, pyridostigmine bromide, and permethrin, in combination with desert dust metals, have subtle adverse effects on blood brain barrier function in an in-vitro model

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Abstract

Aims: In an effort to better understand factors that may play a role in the development of Gulf War Illness, we examine effects of 3 organic compounds and 9 metals of interest to the military population serving during the Gulf War on the function and stability of a model of the human blood brain barrier (BBB).

Main methods: Using a BBB model consisting of human brain endothelial cell and astrocyte cell co-cultures in a transwell membrane system, we exposed transwells to organic compounds and metals either individually or as a combination of an organic followed by a metal and assessed changes in BBB function. Assays included trans-epithelial electrical resistance (TEER), translocation of two different sized fluorescence markers or metals of interest from the luminal side to the abluminal side of the BBB, and changes in expression of tight-junction proteins and cytokines.

Key findings: Several co-exposures resulted in reduced TEER values, although minimal or no effects were seen on transfer of the fluorescent markers and metals across the membrane. Expression of tight junction proteins were also unaffected, but some cytokines increased synergistically with several co-exposures. Of all combinations, pyridostigmine bromide, permethrin, and depleted uranium had the most consistent effects.

Significance: The cause of Gulf War Illness is not well understood, and interactions of pesticides, prophylactics, and metals may factor into symptom development more than any single exposure. Understanding these interactions may also shed light on risk factors for other populations potentially exposed to similar contaminants in their environment.

Key words

Gulf War Illness; pyridostigmine bromide; permethrin; DEET; desert dust; metal; toxicity; blood brain barrier

Introduction

Gulf War Illness (GWI) refers to the chronic multi-symptom illness characterized by cognitive problems, fatigue, and muscle pain affecting close to 700,000 US military personnel who served in the Persian Gulf War in 1990-1991. A variety of exposures to common in-theater chemicals have been suggested for causing GWI, but the large number of personnel affected and the multi-symptom nature of GWI does not fit any single known exposure scenario, suggesting instead a multi-exposure condition [1].

Three organic compounds in particular have surfaced as potential candidates: Pyridostigmine bromide (PB), an investigational nerve gas prophylactic self-administered to military personnel; N,N-Diethyl-*m*-toluamide (DEET), an insecticide/insect repellent spray widely used by the military; and permethrin (PM), a synthetic pesticide spray also commonly used during the Gulf War [2-11]. Alone and if recommended use is followed these chemicals have been shown to be safe, but at higher

levels they can be toxic [12,13] and there is no way to know the actual dose exposure to personnel as this wasn't carefully tracked. PB in particular, alone or in combination with DEET, PM, or stress, could be linked with illnesses in Gulf War veterans, particularly with disruption of the blood brain barrier (BBB) and neurological deficits [14-30].

Another potentially important component comes from respiratory exposure to local fine-grained sand particles, deemed "desert dust," which contained high levels of a variety of metals as well as microbial contaminants [31-35] which were deemed to be exceeding the Military Exposure Guidelines (MEG) over 97% of the time for personnel during Operation Iraqi Freedom and Operation Enduring Freedom [36]. Many studies have shown that internalized metals are capable of crossing the BBB, especially in the presence of other pollutants that increase BBB permeability, and inducing neuronal injury, behavioral changes, and neurological diseases [37-61]. Local populations do not experience GWI symptoms, further supporting a hypothesis of a multi-exposure scenario.

We hypothesize that PB, PM, or DEET adversely affect BBB permeability, allowing inhaled metals to enter the brain and result in neuronal injury. To test this, previously in Hoffman & Kalinich [62] we determined sub-toxic doses of 3 organic compounds (10 μ M) and 9 metals of interest (1 μ M) for human BMEC and astrocyte cell cultures, assessing viability and markers of oxidative stress in response to these compounds alone or in combined exposures. Here, we expand upon this foundation and assess effects on BBB function and inflammatory response using an *in vitro* transwell BMEC and astrocyte co-culture model.

Materials and Methods

Cells and cell culture

Human Astrocytes: Normal Human Astrocytes (male, #1800) were purchased from ScienCell Research Labs (Carlsbad, CA). Cells were maintained in Dulbecco's Minimal Essential Medium (D-MEM) with Glutamax (Gibco, Grand Island, NY) supplemented with N2 Supplement CTS (Gibco) and Fetal Bovine Serum One-Shot (10%, Gibco) and were not used past passage 8 for all experiments.

Human Brain Endothelial Cells: Human Brain Microvascular Endothelial Cells (BMECs, male, ACBRI 376) were obtained from Cell Systems (Kirkland, WA) and cultured in Vasculife VEGF Basal Medium supplemented with the associated VEGF LifeFactors Kit (Lifeline Cell Technology, Frederick, MD) with the following modifications. The LifeFactors Kit fetal bovine serum was not used and only 10 ml of the LifeFactors-provided L-glutamine was used as a supplement. In addition, iCell Endothelial Cell Medium Supplement (Cellular Dynamics International, Madison WI) was added to 10%. Cells were not used past passage 8 for all experiments.

Culture set-up: Stock cell cultures were maintained in tissue culture flasks (75 cm² area) at 37°C in a humidified atmosphere of 5% CO₂. For endothelial cells, the flasks were treated with 6 ml of Quick-Coat (Angio-Proteomie, Boston, MA) to provide a suitable substrate for attachment. For both cell lines, medium was refreshed every other day and cells were subcultured when 70-80% confluent based upon direct microscopic observation. Briefly, medium was aspirated from the flasks and the flasks were washed once with phosphate-buffered saline (PBS, Gibco). After aspirating the PBS, 3 ml of StemPro Accutase Cell Dissociation Reagent (Gibco) were added and the flask left at room temperature for up to 8 min to allow the cells to dissociate from the flask. Once detached, fresh medium was added, and the cell suspension pipetted to break up any clumps. The cell suspension was centrifuged at 240 x g for 10 min after which the cell pellet was resuspended in a suitable amount of the appropriate medium and transferred to new flasks. Cell numbers and viability were determined using trypan blue (Gibco) exclusion with the Bio-Rad Model TC-20 Cell Counter (Hercules, CA).

Organics and metals for treatment

Organic compounds of interest: The following compounds were tested, pyridostigmine bromide (PB), permethrin (PM), and N,N-diethyl-*m*-toluamide (DEET) (all Sigma Chemical, St. Louis,

MO). PB, PM, and DEET were only slightly soluble, if at all, in water. Stock solutions were prepared in dimethylsulfoxide (DMSO, Invitrogen, Carlsbad, CA) before dilution to appropriate working concentrations in cell culture medium.

Metals of interest: Metals to be tested included aluminum (Al as aluminum chloride hydrate), iron (Fe as iron chloride hexahydrate), depleted uranium (DU as uranyl nitrate hexahydrate), nickel (Ni as nickel chloride hexahydrate), cobalt (Co as cobalt chloride hexahydrate), copper (Cu as copper chloride dihydrate), strontium (Sr as strontium chloride hexahydrate), manganese (Mn as manganese chloride tetrahydrate), and zinc (Zn as zinc chloride). All metals were obtained from Sigma Chemical except for uranyl nitrate hexahydrate which was from Fluka Chemical (Ronkonkoma, NY). Metals were dissolved in the appropriate cell culture medium and diluted to provide proper working concentrations.

Microscopy

For images, BMEC cells were plated at 50,000 cells / chamber on Nunc Lab-Tek II 8-chamber slides (Nalgene Nunc, Rochester, NY) coated with Quick-Coat for 5m, and allowed to incubate at 37°C, 5% CO₂ for 24h. Media was then removed and cells treated with media spiked with one of three organic compounds of interest (10 µM) or media control for 24h, then media changed to one of nine metals of interest (1 µM) or media control and incubated for an additional 24h. Medium was aspirated, the chambers washed with Dulbecco's PBS (DPBS, Gibco) and cells fixed with ice-cold methanol for 5m. After removing the methanol, the slides were air dried before being stained with Giemsa (Gibco) stain (1:20 dilution of stock) for 10m followed by extensive washing with tap water. Images were taken with an Olympus BX61 Microscope with D72 camera and cellSens Entry Software (version 1.5) (Olympus America, Melville, NY). Images are presented in Figures S1-S6.

In Vitro Blood-Brain Barrier (BBB) Model System

An *in vitro* system using transwell inserts was used to assess BBB functionality after organic and metal exposures. Briefly, 24-well plates with Corning (Corning, NY) Costar 6.5 mm Inserts with a polyester membrane (0.4 µm pore size) were used to co-culture astrocytes on the abluminal (brain) side and endothelial cells on the luminal (blood) side of the transwell. On the basolateral (abluminal) side of the inverted transwell, 80 µl of a 3.125×10^5 cells/ml suspension were added and the transwells incubated for 4h at 37°C and 5% CO₂. After that time, the plate is flipped and 100 µl of 1×10^6 cells/ml is added to the apical (luminal) side of the transwell. The plates are returned to the incubator for 24h. After that time, as a measure of the integrity of tight junctions and thus the overall health of the cells, Trans-Epithelial Electrical Resistance (TEER) was determined. Measurements were taken prior to addition of the organic compounds, 24h after organic compound addition, and 24h after metal addition. Resistance (R, in ohms, Ω) measurements were taken with the EVOM2 Epithelial Volt-Ohm Meter (World Precision Instruments, Inc., Sarasota, FL) using the STX2 chopsticks electrode to determine the trans-epithelial electrical resistance (TEER) value, which is calculated as $(R_{\text{target}} - R_{\text{blank}}) \times (M_{\text{area}})$, where M_{area} is the membrane area of the transwell (0.33 cm²); TEER units as $\Omega \cdot \text{cm}^2$.

A second assessment of BBB functionality used a modification of previously published procedures [63,64] to determine perturbations of tight junctions by using fluorescently labeled dextran molecules of different molecular weights. In this procedure, transwell plates were prepared as above. At 24h after metal exposure (and 48h after organic exposure), 75 µl of medium from the apical side of the transwell was removed for determination of inflammatory cytokines and 200 µl removed from the basolateral side for metal translocation analysis and replaced with 200 µl of fresh astrocyte medium. The remaining medium on the apical side of the transwell was aspirated, the transwell washed with 100 µl of PBS, and then 100 µl of endothelial medium containing 100 µg/ml of fluorescein (FITC)-labeled dextran (4 kDa, Sigma) and rhodamine (TRITC)-labeled dextran (70 kDa, Sigma) was added and the plates returned to the incubator for 2h. After this time 2 x 100 µl aliquots are removed and placed in wells of a black 96-well plate for fluorescence measurements. Using the BioTek Synergy

Model H1M Multimodal Plate Reader with GEN5 Software, BioTek Instruments (Winooski, VT), excitation/emission values for fluorescein (485 nm / 544 nm) and rhodamine (520 nm / 590 nm), and standard curves, the amount of the 4 kDa and 70 kDa dextran that crossed the *in vitro* blood-brain barrier could be determined.

Metal analysis by inductively coupled plasma mass spectroscopy (ICP-MS)

All compounds used in this study were of the highest grade available. Plastic ware and other disposables were also obtained from Thermo Fisher Scientific. Samples were dissolved in ultrapure nitric acid (Fisher Scientific, Newark, DE) and metal content determined using an inductively coupled plasma-mass spectrometer (XSeries 2 ICP-MS System, ThermoFisher, Madison, WI) equipped with a Cetac ASX520 Autosampler (Cetac Technologies, Omaha, NE). High-pressure liquid argon, 99.997%, was used for the plasma gas. The instrument was calibrated with external standards of the appropriate metal standard (SPEX CertiPrep, Metuchen, NJ) in 2% HNO₃. The sample probe was washed with a constant flow of 2% nitric acid between measurements to prevent carryover. Quantitative analysis was obtained by reference to the slope of the calibration curve (counts per second / ng per liter) as well as an internal standard. ICP-MS operating conditions and parameters can be found in Table S1. Limit of Detection (LoD)/Limit of Quantitation (LoQ), in ppb, are as follows: Al - 0.38/0.44; Fe - 1.08/1.85, Ni - 0.17/0.21; Sr - 0.01/0.05; Co - 0.03/0.06; Cu - 0.24/0.54; Mn - 0.45/0.6; Zn - 2.80/3.01; U - 0.02/0.07.

BBB protein marker and inflammatory cytokine expression

The following procedure was used to provide samples to assess changes in protein expression relevant to BBB function and inflammatory cytokine response of endothelial cells treated with metals after the presence or absence of Gulf War-associated organic compounds. The basic experimental design was to plate the cells on Day 1, replace the medium on Day 2, add organic compounds (if required) on Day 3, add metals (if required) on Day 4, and harvest on Day 5. Briefly, brain microvascular endothelial cells were harvested as above and re-plated on 6-well tissue culture plates at a concentration of 2×10^5 cells/well and the plates returned to the incubator. The next day the medium was aspirated and replaced with fresh medium (2 ml/well). If required, on the next day the appropriate organic compound was added to a non-toxic dose (as determined above) and the plates returned to the incubator. The next day, if required, appropriate metals were added, again at a non-toxic dose as previously determined and the plates returned to the incubator. The next day, 1 ml of medium was removed from each well, placed in a 1.5 ml tube and centrifuged at $18,400 \times g$ for 10 min to pellet any detached cells. The supernatant (0.8 ml) was then removed, placed in a fresh 1.5 ml tube and stored at -80°C until needed for the cytokine assays. The remaining medium in the wells was aspirated and the wells washed with 2 ml of PBS (Gibco). After aspirating the PBS, 1 ml of Accutase was added to each well and the plates incubated at room temperature for 8 min to detach the cells. The cell suspension was placed in a 1.5 ml tube and centrifuged at $18,400 \times g$ for 10 min to pellet the cells. After removing the supernatant, the cell pellets were stored at -80°C until required for protein expression analysis.

Protein expression using the ProteinSimple Wes: Cell pellets were re-suspended in RIPA buffer (Thermo, Waltham, MA, cat#89901) plus Halt Protease and Phosphatase Inhibitor Cocktail (Thermo, Waltham, MA, cat#78442) and homogenized in a Bullet Blender (Next Advance, Troy, NY) with 0.5 mm glass beads (NextAdvance, Troy, NY, cat#GB05-RNA) (settings speed 6, 5 min x 2 runs) and then centrifuged at $1340 \times g$ for 10 min. Total protein from each sample supernatant was measured by Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, cat #500-0006), in triplicate, against a BSA standard curve, on a spectrophotometer (BioTek Synergy Model H1M Multimodal Plate Reader with GEN5 Software), and read at 595 nm. Proteins of interest were quantified using an automated capillary-based size sorting chemiluminescent system 'WES' from ProteinSimple (San Jose, CA). All procedures were performed with manufacturer's reagents (ProteinSimple, 12-235 kDa kit, cat#SM-W004-1 and PS-ST01EZ-8) according to the user manual

with some adjustments: samples are aliquoted to 1 µg/µl before mixing 4 µl with 1 µl fluorescent master mix, then denatured at 94°F (34.4°C) for 4 min, given a quick spin, and loaded on the plate at 4 µl per well. In run settings, stacking time was changed to 18 sec, separation time to 31 min, and antibody diluent time to 30 min. Antibody information is listed in Table S2. Peak values were determined using Compass Software (ProteinSimple). Target proteins are presented as a ratio of the target protein expression normalized to within-sample β-actin expression in arbitrary units. ProteinSimple Wes system uses capillary size separation and chemiluminescent detection, and an example of the raw data and corresponding traditional Western blot image derived from it using the same cell cultures in this publication can be found in our previous publication Hoffman & Kalinich [62] Figure S6.

Cytokine determination using Meso Scale Discovery (MSD): The ability of the various treatments to induce various cytokines was assessed using the U-PLEX Human Biomarker Group 1 kit (Meso Scale Discovery, Rockville, MD). The following biomarkers were assessed in the collected endothelial cell supernatants: IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, Granulocyte Macrophage Colony Stimulating Factor (GM-CSF), and Interferon gamma (IF-γ). The Meso Scale U-PLEX uses linkers to couple to biotinylated capture antibodies. The complexes were self-assembled onto distinct spots on the U-PLEX assay plate. After sample (25 µl of cell supernatant) incubation and washing, an electrochemiluminescent detection antibody was added and the plate read in a Meso Scale Discovery QuickPlex Reader. Concentrations of the various cytokines were determined by comparison to a standard curve.

Statistical analyses

Analyses were performed using GraphPad Prism Software (version 8.4.2, La Jolla, CA). n=4 treated wells per group for all experiments. One-way ANOVAs with Sidak's multiple comparisons post-hoc tests were performed, with specific group comparisons or any other statistical analyses noted. For metal transfer experiments, metal-only transfer values were compared to each corresponding organic+metal group within an organic exposure set using multiple t-tests and a false discovery rate (FDR) approach using the two-stage set-up method of Benjamini, Krieger, and Yekutieli with Q=1%. Specific post-hoc *P* values less than 0.05 were considered significant.

Results

We hypothesize that combined exposures of sub-toxic levels of DEET, PB, or PM adversely affect the viability of cells involved in the BBB, altering its permeability and function, potentially allowing metals solubilized from inhaled desert dust particles to enter the brain. To test this, a transwell BBB model using co-cultures of human BMEC and astrocyte cells were exposed to DEET, PB, or PM, followed by nickel, cobalt, strontium, zinc, manganese, copper, iron, aluminum, or depleted uranium and assessed for any changes in function and cytokine response.

Trans-Epithelial Electrical Resistance (TEER) after exposure to organic and metal compounds

One of the major functions of the blood brain barrier is to prevent passive transport of compounds from the blood to the interstitial space of the brain. One way to measure this function is by measuring the Trans-Epithelial Electrical Resistance (TEER), which is representative of how tight of a barrier the BBB has formed between cells. The higher the measured resistance, the less passive transport is expected to occur. Using an *in-vitro* model of the BBB, the co-culturing of BMEC and astrocytes in a transwell plate, cells were grown over the course of several days and treated concurrently to account for changes over time in tight junction formation. The first two days all cells received media alone to allow for optimal tight junction growth. The next day the media only group received only media, and all other groups received media with the indicated organic. On the third day the media only group received only media, the organic only group received media with the indicated organic, and the organic plus metal group received media with the indicated organic and metal co-

exposure. Although TEER readings were completed each day before media change, data presented here is from the final reading, with values normalized to a set of wells with no cell culture to control for resistance values of the membrane alone. Analysis is presented as a one-way ANOVA with Sidak multiple comparisons tests specifically for the media-only control vs the organic alone (i.e. effect of organic), and then the organic alone vs each organic plus metal group (i.e. does the metal have any effect beyond exposure to the organic compound) (Figure 1: A) DEET plus metals, ($F_{(10,153)}=4.913$, $p<0.0001$); B) PB plus metals, ($F_{(10,153)}=5.50$, $p<0.0001$); DEET plus metals, ($F_{(10,153)}=9.825$, $p<0.0001$).

DEET, PB, and PM alone had no significant effect on the TEER reading compared to the media-only control. DEET + Cu exposure significantly increased the TEER value over DEET alone ($*p=0.0039$), suggesting a possible protective effect. PB + DU exposure significantly decreased the TEER value over PB alone ($*p<0.0001$), suggesting DU may work synergistically with PB to make the BBB more leaky. Multiple metals had significant effects on TEER when combined with PM compared to PM alone. Fe increased TEER value ($*p=0.0004$), while Sr, Mn, Zn, and DU all decreased TEER value ($*p=0.0092$, $*p=0.0179$, $*p=0.0005$, and $*p=0.0012$, respectively).

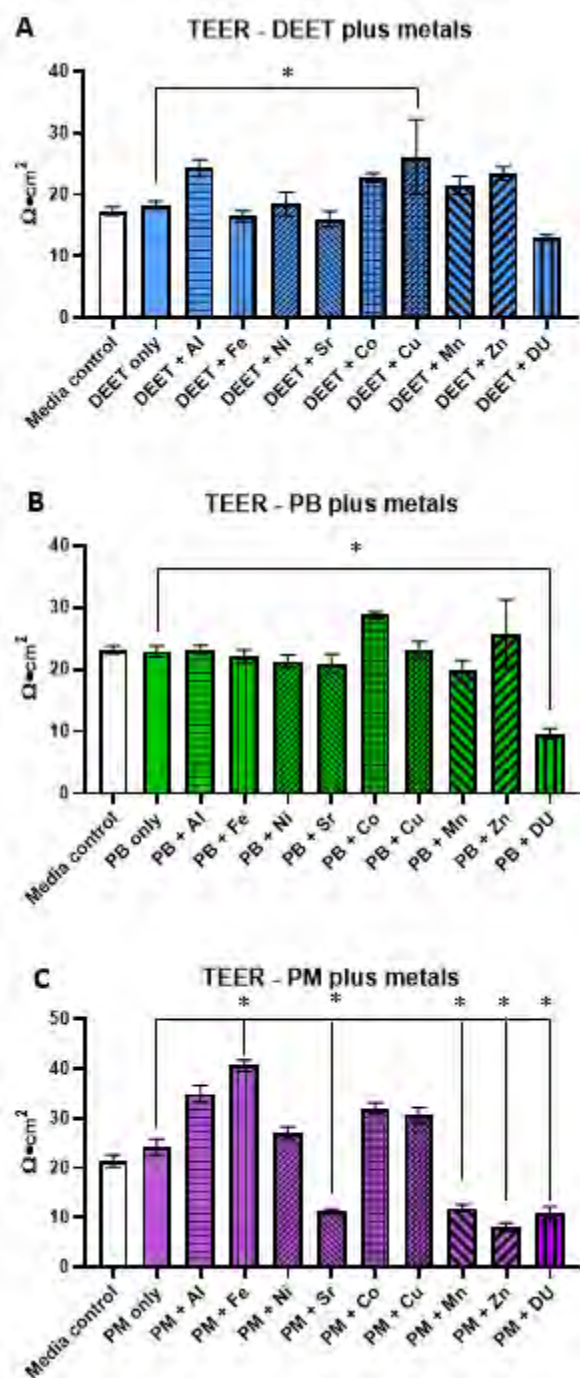


Figure 1: Trans-epithelial electrical resistance (TEER) of a BBB transwell model after exposure to organic and metal compounds. TEER values of resistance across a membrane with co-cultures of BMEC and astrocytes given in $\Omega \bullet \text{cm}^2$. White bar represents media only control. Colored bars indicate organic treatment, pattern indicates subsequent metal treatment with no pattern indicating media only instead of metal. A) DEET (blue) plus metals, B) PB (green) plus metals, C) PM (purple) plus metals. * indicates significant p-value for comparison between groups indicated with connected lines.

Fluorescence transfer after exposure to organic and metal compounds

Tracking movement of different sized fluorescent molecules from the endothelial side of a BBB transwell model to the astrocyte side of the transwell can indicate if the integrity of the BBB is intact or compromised and leaky. BMEC and astrocytes were co-cultured in a transwell setup and exposed to a media-only control, each metal alone, an organic (DEET, PM, or PB) alone, or the organic plus each individual metal. A no-cell well condition was also included as a baseline for how much

fluorescence transfer is obstructed by the transwell membrane alone. FITC dextran (4 kDa) or TRITC dextran (70 kDa) was then introduced to the endothelial side, and after incubation, the amount of fluorescence signal was measured in the astrocyte side. Analysis is presented as a one-way ANOVA with Sidak multiple comparisons tests specifically for the no-cell control vs media only control (i.e. the baseline of how much fluorescence is prevented from crossing with the BMEC and astrocyte co-culture in place), the media-only control vs the metal alone or organic alone (i.e. effect of metal or organic), and then the organic alone vs each organic plus metal group (i.e. does the metal have any effect beyond exposure to the organic compound) (Figure S7: A) 4 kDa FITC, metals alone, $F_{(10,33)}=28.65$, $p<0.0001$; B) 70 kDa TRITC, metals alone, $F_{(10,33)}=20.69$, $p<0.0001$; C) 4 kDa FITC, DEET plus metals, $F_{(11,36)}=74.77$, $p<0.0001$; D) 70 kDa TRITC, DEET plus metals, $F_{(11,36)}=79.83$, $p<0.0001$; E) 4 kDa FITC, PB plus metals, $F_{(11,36)}=34.60$, $p<0.0001$; F) 70 kDa TRITC, PB plus metals, $F_{(11,36)}=31.01$, $p<0.0001$; G) 4 kDa FITC, PM plus metals, $F_{(11,36)}=128.5$, $p<0.0001$; H) 70 kDa TRITC, PM plus metals, $F_{(11,36)}=86.67$, $p<0.0001$).

For both sizes of fluorescent compound in all treatment experiments, the media-only control prevented fluorescent signal from passing across the membrane compared to the no-cell control (* $p<0.0001$ for all eight sets). None of the metals and none of the organics (DEET, PB, and PM) alone had any significant effect on fluorescence transfer compared to the media only control. Additionally, there were no combinations of an organic compound plus metal treatment compared to the corresponding organic compound alone with a significant effect on fluorescence control, suggesting no effect of organic compounds or organic plus metal exposure on the integrity of the BBB. We also note that only around half the 4 kDa FITC dextran was prevented from crossing the membrane by the media-only cell control compared to the no cell control, whereas all of the larger 70 kDa TRITC dextran was prevented from crossing the barrier.

Metal transfer after exposure to organic and metal compounds

Similar to the fluorescence transfer, we wanted to assess how much of each metal was able to cross the BBB in conjunction with exposure to the organic compounds of interest. Transwells were set up with co-cultures of BMECs and astrocytes and exposed to 1 μ M of a known amount of metal on the BMEC side of the transwell following exposure to each organic of interest or media alone to determine if exposure to the organic compound facilitated metal translocation. Metal concentration was measured on the astrocyte side of the transwell, and presented as a percentage of the total metal exposure (% translocated metal), presented in Figure 2. Of the metal-only exposures, all metals crossed the membrane to varying degrees with the exception of Fe, which did not transfer at all (one-way t-test comparing % translocated metal to a value of 0; Fe no detectable metal crossed, Zn * $p=0.0131$, Al * $p=0.004$, DU * $p=0.0003$, all other metals * $p<0.0001$). The metal-only exposure groups served as the control for each of the organic+metal comparisons.

Surprisingly, for all three organic+metal experimental sets, transfer of Cu, Co, and DU were decreased after each organic exposure compared to the metal exposure alone (DEET Figure 2A: Cu t-ratio=5.079, * $p=0.0023$; Co t-ratio=9.667, * $p<0.0001$; DU t-ratio=11.32, * $p<0.0001$; PB Figure 2B: Cu t-ratio=4.527, * $p=0.004$; Co t-ratio=6.724, * $p=0.0005$; DU t-ratio=12.27, * $p<0.0001$; PM Figure 2C: Cu t-ratio=9.053, * $p=0.0001$; Co t-ratio=7.668, * $p=0.0003$; DU t-ratio=12.12, * $p<0.0001$). Additionally, Mn transfer across the membrane was also decreased when in combination with both PB (Figure 2B: t-ratio=4.468, * $p=0.0043$) and PM (Figure 2C: t-ratio=9.602, * $p=0.0001$) compared to Mn transfer alone. Al transfer was increased after exposure to PM (Figure 2C: t-ratio 5.839, * $p=0.0009$). It is also of interest to note that although it did not reach statistical significance, Fe transfer across the membrane also increased from 0% to 2.53% after exposure to PM.

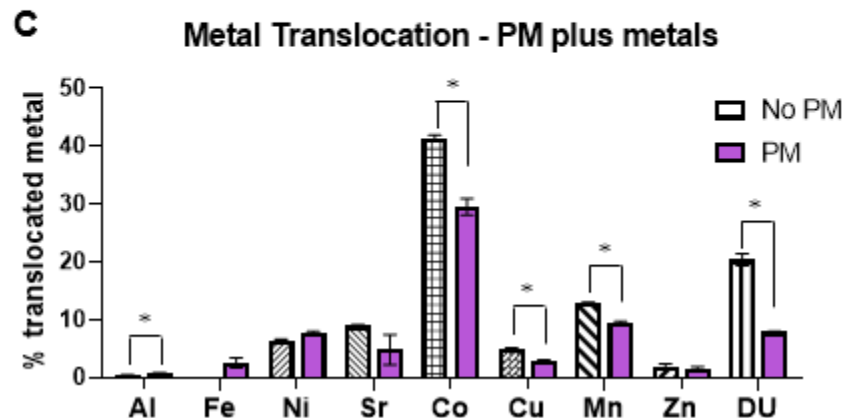
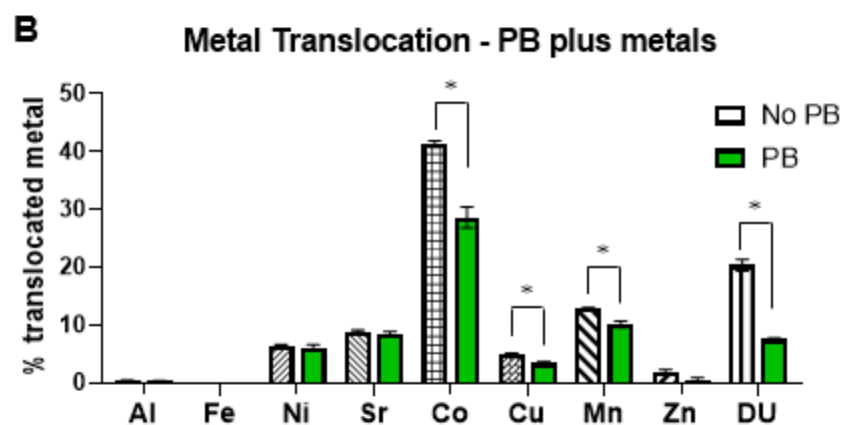
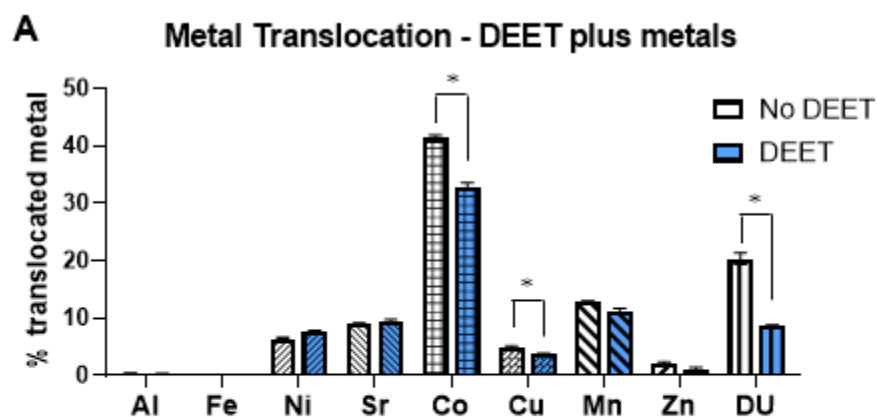


Figure 2: Transfer of metals across a BBB transwell model after exposure to organic and metal compounds. Data presented as percent of translocated metal to the astrocyte side of the BBB model transwell from a known concentration presented to the endothelial side after exposure to media or organic compounds. White bar represents media only control. Colored bars indicate organic treatment, pattern indicates subsequent metal treatment. A) DEET (blue) plus metals, B) PB (green) plus metals, C) PM (purple) plus metals. * indicates significant p-value for comparison between groups indicated with connected lines.

BBB tight junction protein expression after exposure to organic and metal compounds

We examined the effects of organic and metal compound exposure to BMEC cell cultures on expression of BBB related proteins, occludin, ZO-1, and claudin 5 because of their function in BBB tight junction formation and thus, permeability [65]. In each experimental set, BMECs were cultured alone and exposed to a media control, an organic compound alone, or the organic compound in

combination with one of the metals. Statistical analysis is presented as a one-way ANOVA of the entire set, with post-hoc Sidak's multiple comparisons test comparing media to the organic compound alone, to determine the effect of the organic compound, followed by individual comparisons of the organic compound versus the organic plus each individual metal, to determine any further effects of metal exposure. Graphs are presented as arbitrary units, where the chemiluminescence expression level of the target protein is normalized to the expression level of β -actin within each sample.

For the DEET + metals experimental set, there were no significant differences in expression of any protein after any treatment, with the exception of DEET + Fe compared to DEET alone on occludin (Figure S8A) ANOVA $F_{(10,32)}=1.658$, $p=0.1347$, Sidak comparison $p=0.0348$). Occludin expression would be expected to decrease if the BBB were disrupted, but DEET + Fe actually resulted in a higher expression of occludin than DEET alone, suggesting a possible protective effect, or possibly overexpression to compensate for a loss of function. All protein results are shown in Figure S8 (B) ZO-1, $F_{(10,33)}=2.126$, $p=0.0507$; C) claudin 5, $F_{(10,33)}=0.4763$, $p=0.8933$). For the PB + metals experimental set, there were no significant differences in expression of any protein after any treatment. All protein results are shown in Figure S9 (A) occludin, $F_{(10,32)}=1.692$, $p=0.1258$; B) ZO-1, $F_{(10,33)}=0.3921$, $p=0.9409$; C) claudin 5, $F_{(10,33)}=0.2610$, $p=0.9856$). For the PM + metals experimental set, there were also no significant differences in expression of any protein after any treatment. All protein results are shown in Figure S10 (A) occludin, $F_{(10,33)}=0.6181$, $p=0.7874$; B) ZO-1, $F_{(10,32)}=0.3662$, $p=0.9524$; C) claudin 5, $F_{(10,32)}=0.1738$, $p=0.9971$).

Cytokine expression after exposure to organic and metal compounds

Similar to the protein expression experiments, we also examined effects of organic and metal compound exposure on changes in 9 cytokines in BMEC cultures (IL-2, IL-8, IL-12p70, IL-1 β , GM-CSF, IFN- γ , IL-6, IL-10, and TNF- α). In each experimental set, BMECs were cultured alone and exposed to a media control, a metal alone, an organic compound alone, or the organic compound in combination with one of the metals. Statistical analysis is presented as a one-way ANOVA of the entire set, with post-hoc Sidak's multiple comparisons test comparing media to the organic compound alone and individual comparisons of the organic compound versus the organic plus each individual metal.

For the metals alone experimental set all cytokine results are shown in Figure 3 (A) IL-2, $F_{(9,20)}=3.784$, $p=0.0063$; B) IL-8, $F_{(9,20)}=4.399$, $p=0.0028$; C) IL-12p70, $F_{(9,20)}=21.79$, $p<0.0001$; D) IL-1 β , $F_{(9,20)}=1.688$, $p=0.1577$; E) GM-CSF, $F_{(9,20)}=1.325$, $p=0.2854$; F) IFN- γ , $F_{(9,20)}=2.494$, $p=0.0426$; G) IL-6, $F_{(9,20)}=729.9$, $p<0.0001$; H) IL-10, $F_{(9,20)}=2.463$, $p=0.0447$; I) TNF- α , $F_{(9,20)}=1.316$, $p=0.2896$). Compared to media alone, the only metal alone with no effect on IL-2 was Sr. All other metals alone statistically decreased IL-2 levels: Al * $p=0.0020$, Fe * $p=0.0046$, Ni * $p=0.0021$, Co * $p=0.0008$, Cu * $p=0.0234$, Mn * $p=0.0186$, Zn * $p=0.0294$, and DU * $p=0.0461$. For IL-8, Zn and DU had no effect compared to media alone, while the rest of the metals decreased IL-8: Al * $p=0.0047$, Fe * $p=0.0064$, Ni * $p=0.0029$, Sr * $p=0.0481$, Co * $p=0.0414$, Cu * $p=0.0103$, and Mn * $p=0.0481$. IFN- γ was increased only by DU exposure compared to media only control (* $p=0.0060$). All metals decreased expression of IL-12p70 and IL-6 compared to media (* $p<0.0001$ for all). IL-1 β , GM-CSF, IL-10, and TNF- α expression were not significantly affected by any metal. Taken together would suggest a subtle decrease in cytokine response regardless of specific metal.

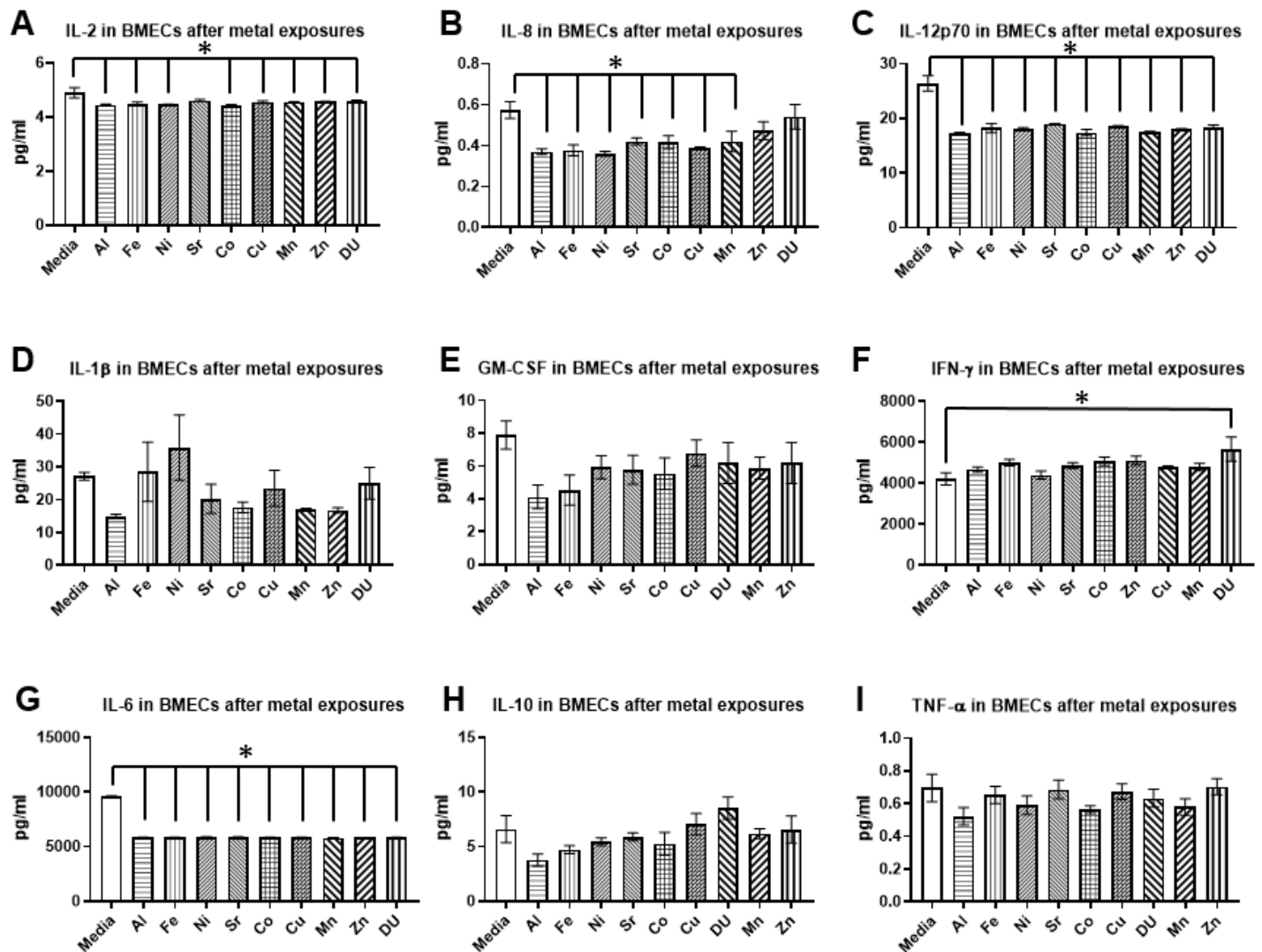


Figure 3: Cytokines in BMEC cultures after exposure to metals alone. Data presented as pg/ml of target cytokine. Bars are white to indicate media only treatments with or without metals. Pattern indicates subsequent metal treatment with no pattern indicating media only instead of metal. A) IL-2, B) IL-8, C) IL-12p70, D) IL-1 β , E) GM-CSF, F) IFN- γ , G) IL-6, H) IL-10, I) TNF- α . * indicates significant p-value for comparison between groups indicated with connected lines.

For the DEET + metals experimental set all cytokine results are shown in Figure 4 (A) IL-2, $F_{(10,22)}=0.4218$, $p=0.9207$; B) IL-8, $F_{(10,21)}=2.747$, $p=0.0245$; C) IL-12p70, $F_{(10,22)}=1.232$, $p=0.3250$; D) IL-1 β , $F_{(10,22)}=0.5463$, $p=0.8386$; E) GM-CSF, $F_{(10,22)}=0.6357$, $p=0.7681$; F) IFN- γ , $F_{(10,21)}=0.9052$, $p=0.5454$; G) IL-6, $F_{(10,22)}=3.264$, $p=0.0099$; H) IL-10, $F_{(10,21)}=0.7160$, $p=0.7008$; I) TNF- α , $F_{(10,22)}=1.481$, $p=0.2119$). DEET alone had no effect on expression of any of the cytokines. In conjunction with DEET, compared to DEET alone, there were no significant changes in cytokine expression from DEET + metal exposures. We would expect to see differences due to effects seen from exposure to metals alone, which may indicate a dampening effect of DEET exposure prior to metal exposure, and thus a possible synergistic increase in cytokine response regardless of metal.

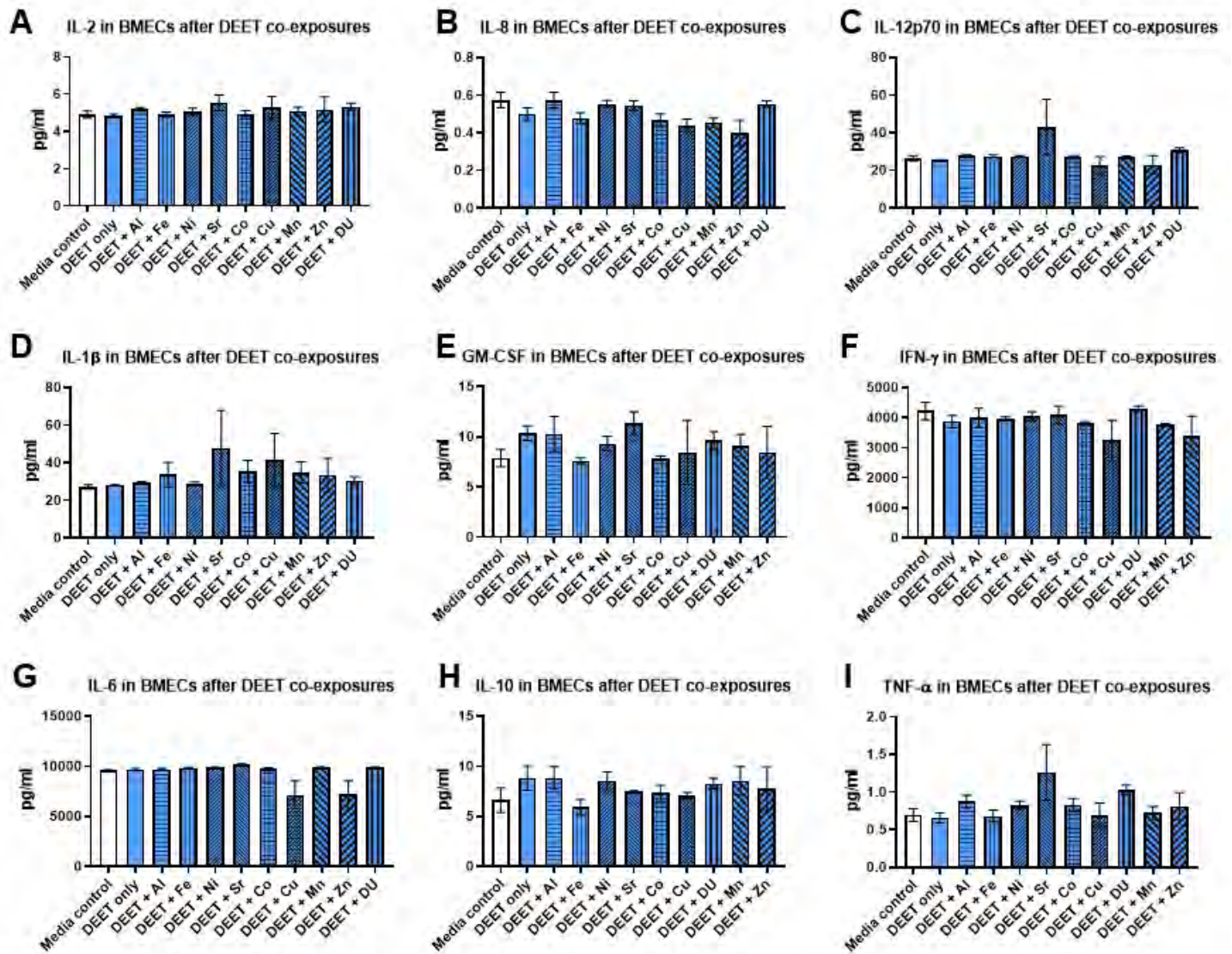


Figure 4: Cytokines in BMEC cultures after exposure to DEET and metal compounds. Data presented as pg/ml of target cytokine. White bar represents media only control. Blue bars indicate DEET treatment, and pattern indicates subsequent metal treatment with no pattern indicating media only instead of metal. A) IL-2, B) IL-8, C) IL-12p70, D) IL-1 β , E) GM-CSF, F) IFN- γ , G) IL-6, H) IL-10, I) TNF- α . No significant differences.

For the PB + metals experimental set all cytokine results are shown in Figure 5 (A) IL-2, $F_{(10,22)}=2.011$, $p=0.0828$; B) IL-8, $F_{(10,22)}=3.286$, $p=0.0096$; C) IL-12p70, $F_{(10,22)}=5.796$, $p=0.0003$; D) IL-1 β , $F_{(10,21)}=1.250$, $p=0.3180$; E) GM-CSF, $F_{(10,22)}=0.8838$, $p=0.5618$; F) IFN- γ , $F_{(10,22)}=6.457$, $p=0.0001$; G) IL-6, $F_{(10,22)}=4.301$, $p=0.0021$; H) IL-10, $F_{(10,22)}=2.717$, $p=0.0243$; I) TNF- α , $F_{(10,22)}=4.834$, $p=0.0010$). PB alone had no effects on any cytokines. In conjunction with PB, compared to PB alone, Ni increased TNF- α ($*p=0.0388$). Cu decreased IFN- γ and IL-6 ($*p=0.0018$ and $*p=0.0283$, respectively). Mn increased TNF- α ($*p=0.0438$). Zn decreased IL-8, IL-12p70, IFN- γ , and IL-6 ($*p=0.0086$, $*p=0.0273$, $*p=0.0003$, and $*p=0.0078$, respectively). There were no other significant changes in cytokine expression from PB + metal exposures. When considering these effects against the metal only exposures, DU exposure alone had increased IFN- γ , but after exposure to PB the effect of DU was no longer significant, indicating a dampening effect of this co-exposure. However, PB had a synergistic decrease in IFN- γ for exposure to Cu and Zn following exposure to PB while the metals alone had no effect. All metals alone had decreased IL-6 and IL-12p70 compared to

media alone, but PB exposure had a dampening effect, where only Cu and Zn following PB exposure significantly reduced IL-6, and only Zn following PB reduced IL-12p70, but the effects in all three cases were reduced. Similarly, IL-2 and IL-8 were decreased by most metal exposures alone, but following exposure to PB this effect was prevented, with no significant changes in IL-2 or IL-8 in any metal after PB exposure that had been significant in metal only exposures. However, although Zn alone had no effect on IL-8 it decreased IL-8 following exposure to PB, indicating a synergistic effect of PB and Zn co-exposure. Metals alone had no effect on TNF- α , but the increase in TNF- α from both Ni and Mn after exposure to PB indicates a synergistic effect of this co-exposure as well. Overall, PB exposure prior to subsequent metal exposure prevented many decreases in the cytokines measured, as well as increasing TNF- α in two metals that had no effect alone, suggesting a synergistic effect of PB and metals on increasing TNF- α inflammatory response.

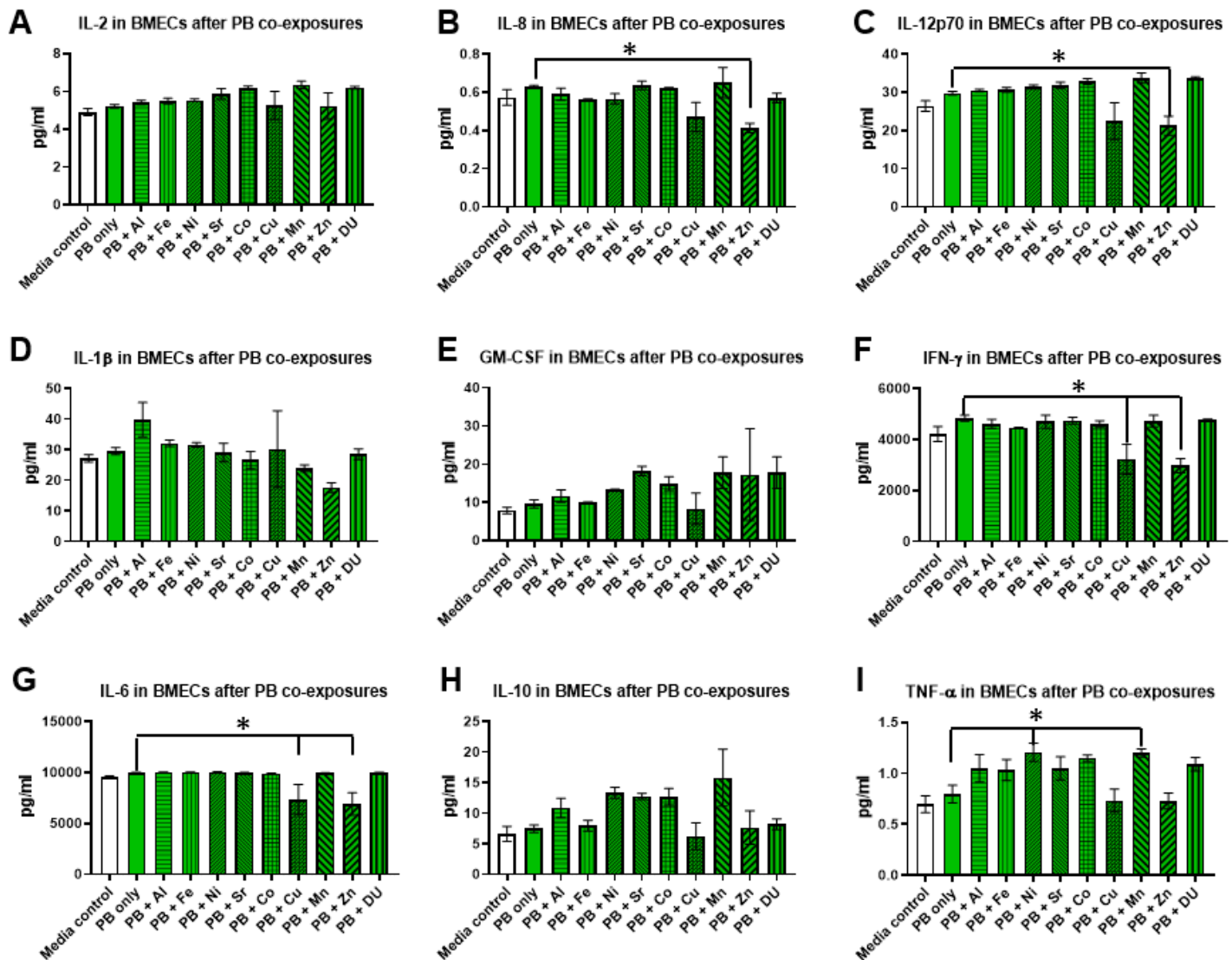


Figure 5: Cytokines in BMEC cultures after exposure to PB and metal compounds. Data presented as pg/ml of target cytokine. White bar represents media only control. Green bars indicate PB treatment, and pattern indicates subsequent metal treatment with no pattern indicating media only instead of metal. A) IL-2, B) IL-8, C) IL-12p70, D) IL-1 β , E) GM-CSF, F) IFN- γ , G) IL-6, H) IL-10, I) TNF- α . * indicates significant p-value for comparison between groups indicated with connected lines.

For the PM + metals experimental set all cytokine results are shown in Figure 6 (A) IL-2, $F_{(10,22)}=7.077$, $p<0.0001$; B) IL-8, $F_{(10,22)}=16.28$, $p<0.0001$; C) IL-12p70, $F_{(10,22)}=14.35$, $p<0.0001$; D) IL-1 β , $F_{(10,21)}=3.835$, $p=0.0046$; E) GM-CSF, $F_{(10,21)}=5.026$, $p=0.0009$; F) IFN- γ , $F_{(10,22)}=26.00$, $p<0.0001$; G) IL-6, $F_{(10,22)}=11.04$, $p<0.0001$; H) IL-10, $F_{(10,22)}=1.051$, $p=0.4367$; I) TNF- α , $F_{(10,22)}=2.510$, $p=0.0346$). PM alone increased expression of IL-2 ($*p=0.0340$), compared to media control, but had no effects on any other cytokines. In conjunction with PM, compared to PM alone, Cu decreased IL-12p70, GM-CSF, IFN- γ , and IL-6 ($*p=0.0002$, $*p=0.0234$, $*p<0.0001$, and $*p=0.001$, respectively). Zn reversed the effect of PM alone and decreased IL-2 ($*p=0.0014$), and decreased IL-12p70, GM-CSF, IFN- γ , and IL-6 ($*p<0.0001$, $*p=0.0179$, $*p=0.0002$, and $*p<0.0001$, respectively). DU increased IL-8, IL-1 β , and IFN- γ ($*p<0.0001$, $*p=0.0013$, and $*p=0.0002$, respectively). There were no other significant changes in cytokine expression from PM + metal exposures. When considering these effects against the metal only exposures, PM alone increased expression of IL-2 compared to the media-only control, but expression remained high for all metals except Zn, which returned to the level of Zn exposure alone. Since Al, Fe, Ni, Co, Cu, Mn, and DU had decreased IL-2 expression, PM therefore overrode the effects of these metals alone. PM exposure prior to subsequent metal exposures prevented the decrease in IL-8 seen in many metal alone exposures. Similarly, PM also prevented the decrease in IL-12p70 and IL-6 seen after all metals except for Cu and Zn. Exposure to Cu and Zn after exposure to PM resulted in a decrease in GM-CSF and IFN- γ that was not seen after the metals alone for either cytokine. DU alone had no effect on IL-1 β or IL-8, but caused an increase in both cytokines following exposure to PM. PM exposure also resulted in a further increase in IFN- γ than DU alone. Overall many effects of metals alone were reversed, and several other cytokines were increased after co-exposure with PM, suggesting a synergistic effect of PM and metals on increasing cytokine response.

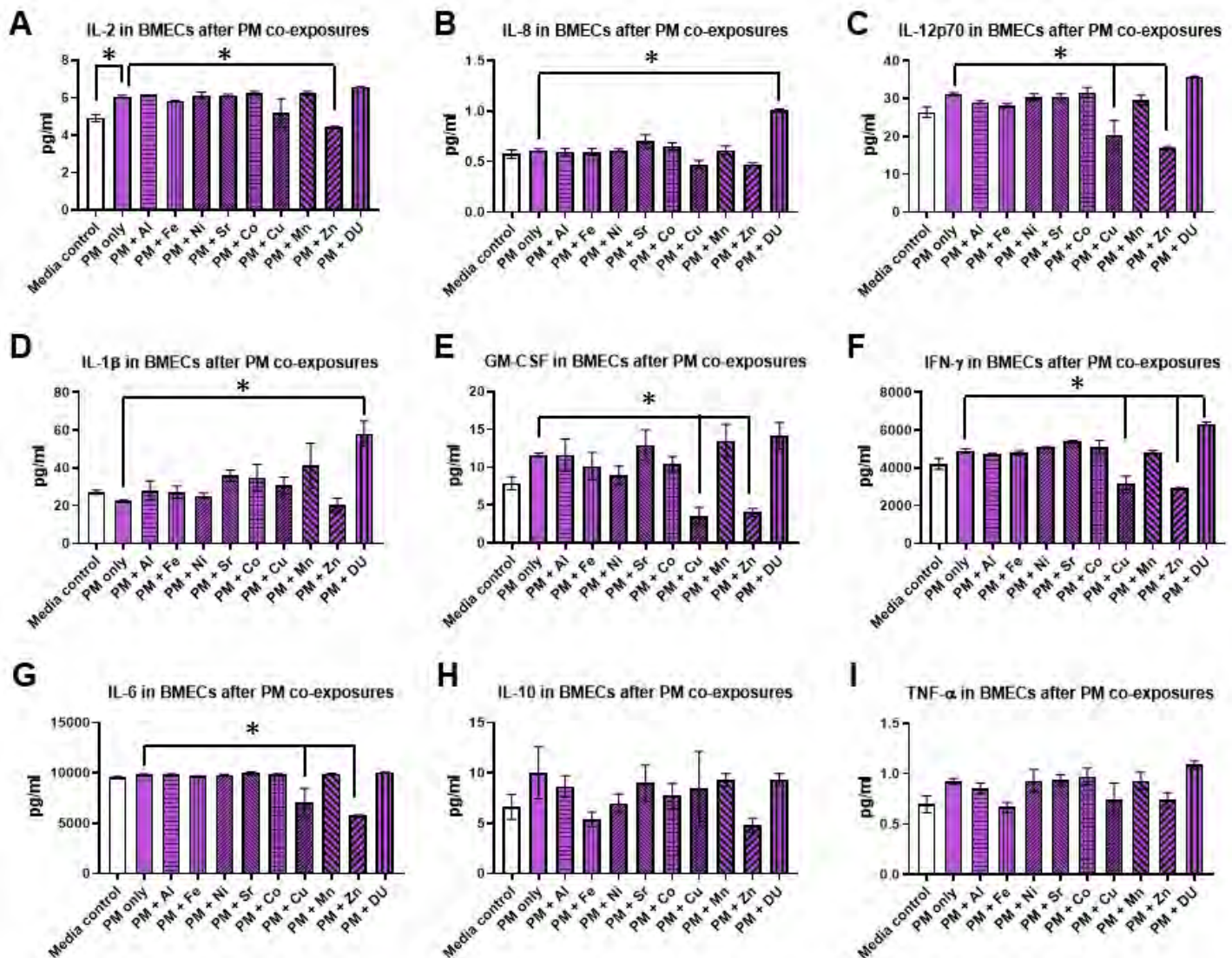


Figure 6: Cytokines in BMEC cultures after exposure to PM and metal compounds. Data presented as pg/ml of target cytokine. White bar represents media only control. Purple bars indicate PM treatment, and pattern indicates subsequent metal treatment with no pattern indicating media only instead of metal. A) IL-2, B) IL-8, C) IL-12p70, D) IL-1 β , E) GM-CSF, F) IFN- γ , G) IL-6, H) IL-10, I) TNF- α . * indicates significant p-value for comparison between groups indicated with connected lines.

Discussion

Currently, the multi-symptom syndrome described as Gulf War Illness, which applies to many US military personnel that served in Persian Gulf War in 1990-1991, has no known single-exposure scenario that applies to everyone diagnosed with GWI. Research instead suggests multiple factors in combined exposure scenarios has created the complex GWI symptom profile. One proposed set of factors is exposure to organic compounds in theatre at the time, such as DEET, pyridostigmine bromide, or permethrin, as well as metals and other microbes internalized through inhalation of contaminated “desert dust” due to their potential to disrupt the blood brain barrier, which could then contribute to symptoms such as fatigue, anxiety, depression, and other cognitive problems seen in GWI. The BBB functions to heavily restrict movement of molecules from the circulatory system into the central nervous system primarily through formations of tight junctions between endothelial cells and supported by astrocytes [65-67]. While there is no way to really know what doses personnel were exposed to, especially when it comes to metals, microbes, any other contaminants inhaled within

desert dust, and actual versus recommended use of pesticides and prophylactics, we decided to work from the assumption of low dose exposures to several compounds of interest rather than overtly high acute exposures. Therefore we hypothesized that exposures of sub-toxic levels of DEET, PB, and PM, particularly in combination with concurrent exposure to sub-toxic levels of metals, would adversely affect the health and viability of cells involved in the BBB, which could lead to subtle changes in permeability and function.

Previously we examined a range of doses of the organic compounds and metals of interest to GWI and their effects on toxicity and viability on human BMEC and astrocyte cell cultures to establish a model of sub-toxic exposure for co-exposure conditions [62]. Not all compounds had the same toxicity profile across the entire dose range tested – for example, Sr did not appear toxic even at high levels. This may affect actual multi-exposure results. In mono-cultures of BMECs, we reported that Co and Mn in co-exposures with all three organics resulted in greater cell toxicity. DU increased nitric oxide (NO, oxidative stress marker) in BMEC cultures regardless of organic co-exposure, but Sr reduced NO levels in co-exposures with all three organics even though it had no effect alone, possibly a protective interaction between the organic compounds and Sr, especially considering Sr's lack of toxicity across investigated doses. We also reported no effects on other oxidative stress markers such as thiobarbituric acid reactive substances (TBARS), a byproduct of lipid peroxidation, protein carbonyl, or multiple protein markers (GPX4, catalase, SOD, MMP3, MMP9).

Here we wanted to investigate effects of DEET, PB, PM, nine metals of interest, and whether a multi-exposure scenario of an organic followed by a metal on more functionally relevant aspects of the BBB, and did so by using human BMECs and human astrocytes co-cultured on a transwell membrane. In examining several markers of BBB function, we found that the transwell model did create an effective barrier to 4 kDa and 70 kDa fluorescence markers, regardless of organic, metal, or combined exposures. TEER, another marker of BBB functionality, was unaffected by DEET, PB, and PM alone at the experimental dose. DEET followed by Cu and PM followed by Fe increased TEER, which may reflect a protective effect, but multiple metals reduced TEER after exposure to PM. In particular, DU decreased TEER values, and thus decreased BBB functionality, after exposure to PM or PB. We were also interested in examining whether organic and metal combination exposures changed the ability of metals to cross over a BBB model. In the absence of organic compound exposure, all metals crossed the membrane to varying degrees with the exception of Fe, which did not transfer at all. Whether this is a result of Fe binding to the serum proteins used to supplement the BMEC growth medium is not known at this time. Surprisingly, metal translocation of several metals actually decreased after exposure to organic compounds, which is the opposite of what we predicted. Al was the only metal to significantly increase translocation across the BBB, and only after exposure to PM compared to Al alone. Given that Fe did not cross the BBB on its own at all, it is interesting to note that it did have a small amount of transfer after exposure to PM, although this did not reach statistical significance. Although the BBB tightly regulates which compounds can move from the circulatory system to the central nervous system, transport mechanisms exist to translocate essential metals across the BBB in order to maintain metal homeostasis. In most cases these metal transporters are capable of translocating more than one metal across the BBB and more than one mechanism exists for the translocation of most essential metals. For example, divalent metal transporter-1 (DMT1) is capable of moving Fe across the BBB but can also transport Cu, Mn, and Al [68]. However, Fe can also be transported with the transferrin and melanotransferrin systems, Cu with copper transporter-1 (CTR1), and both Zn and Mn with the ZIP transporter system [69]. Metallothionein has also been shown to transport both Zn and Cu across the BBB [70]. It is not currently known what transport mechanism would be involved in moving Ni, Sr, and Ni across the BBB since they are non-essential metals with U and Ni in particular having no known biological function. While the ability of the 9 individual metals with and without pre-exposure to organic compounds to cross the BBB was assessed, it is not known whether metals were also transported into the BMECs and sequestered there potentially affecting cell signalling and oxidative stress pathways.

No changes were observed in expression levels of BBB tight junction proteins occludin, ZO-1, or claudin 5, with the exception of DEET + Fe, which increased occludin over DEET alone and yet again suggests potential protective interactive effects involving Fe. It is important to note, however, that lack of change in expression level does not necessarily mean no change in function – we did not examine any changes in protein structure or location changes in the cell membrane versus cytoplasm.

Cytokines represent a broad category of proteins involved in a variety of functions including acute and chronic inflammation. They can be loosely grouped depending on whether they act on the cells of the adaptive immune system, are pro-inflammatory, or are anti-inflammatory [71]. More specifically, the cytokines we assessed can be grouped as follows: adaptive immune response (IL-2, GM-CSF); pro-inflammatory (IL-8, IL-1 β , IFN- γ , IL-6, TNF- α); anti-inflammatory (IL-10, IL-12p70). Metal exposure suggests a subtle decrease in cytokine response regardless of specific metal. For most metals, exposure to DEET, PB, and PM prior to metal exposure prevented the decrease in cytokines IL-2, IL-8, IL-12p70 and IL-6, although this effect was blunted in Cu and or Zn with PB and PM, but not DEET. PB had a synergistic effect with Ni and Mn to increase TNF- α , and PM synergistically increased IL-8, IL-1 β , and IFN- γ when given prior to DU exposure compared with DU metal exposure alone. Interestingly, PM was the only organic compound to have any effect on cytokine expression, increasing IL-2. Overall, sub-toxic doses of all three organic compounds investigated, along with sub-toxic doses of most metals, had little to no effect on cytokine response, with several exceptions. Both PB and PM in combination with Cu or Zn lowered the response of several pro- and anti-inflammatory cytokines. Conversely, DU increased the levels of several pro-inflammatory cytokines when co-exposed with PM, combined with our previous findings that DU had the most impact on human BMEC oxidative stress response compared with other metals and the organic compounds tested, DU continues to be a metal to watch alone and in organic compound combined exposure scenarios.

Military personnel are not the only people facing health effects from exposure to metals and pesticides – contamination of food and water [72-76] or inhalation of metals and other chemicals from e-cigarettes and air pollution [77-79] can affect populations all over the world. Understanding how exposure interactions can affect health in addition to understanding the consequences of exposure to any individual metal or compound is necessary to improve the health and well-being of people in many circumstances.

Conclusion

Our hypothesis that combined exposures of military-relevant organic compounds and metals would lead to synergistic negative effects on human BMEC and human astrocyte cultures and disrupt their function in a BBB model was simple and straightforward, but the effects we found are more complicated. Overall there do seem to be some synergistic effects of organics and metals on functionality of the BBB, particularly PM with multiple metals and DU alone and with multiple organics, but functional changes are subtle. Future studies using a more adaptive models of BBB such as organ-on-a-chip technologies that incorporate flow simulation and more cell types may be better suited to parsing out clinically-relevant changes from exposure to these compounds.

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Author Contributions

JFK was awarded the funding; JFK and JFH designed the experiments; JFK, JFH, AXF, and VBV conducted cell culture maintenance and experiments; JFK, JFH, AXF, and VBV conducted assay data collection; JFH conducted statistical analyses; JFH wrote the original manuscript draft; JFK, JFH, and VBV provided review and editing. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analysis, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Supplementary Materials

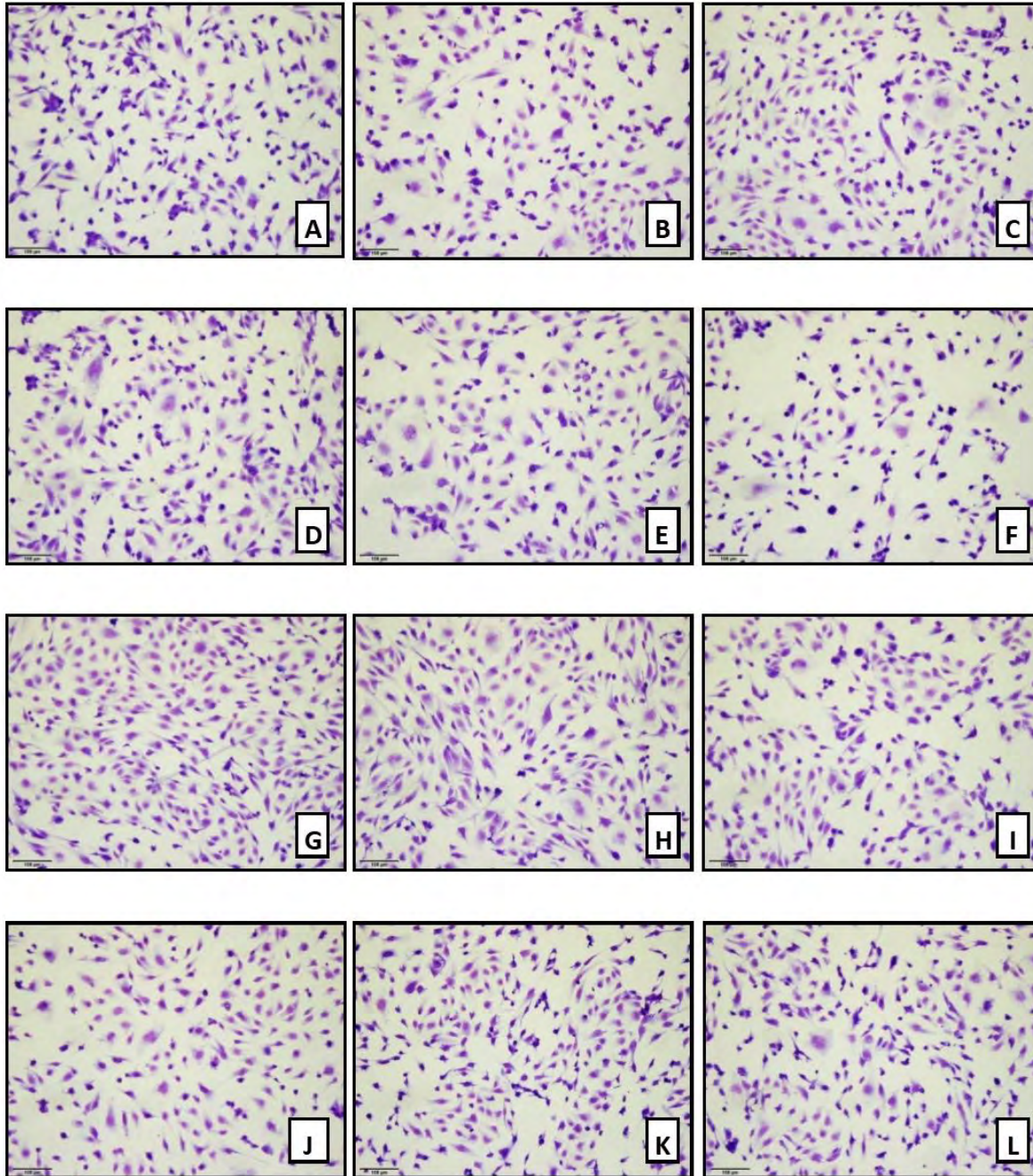


Figure S1: Photomicrographs (10X) of BMEC's treated with DEET (10 μ M) plus/minus metals (1 μ M). BMEC monocultures stained with Giemsa after treatments. A) Control; B) DMSO Control; C) DEET; D) DEET + Al; E) DEET + Fe; F) DEET + Ni; G) DEET + Sr; H) DEET + Co; I) DEET + Cu; J) DEET + Mn; K) DEET + Zn; L) DEET + DU. Magnification 10X, scale bar represents 100 μ m.

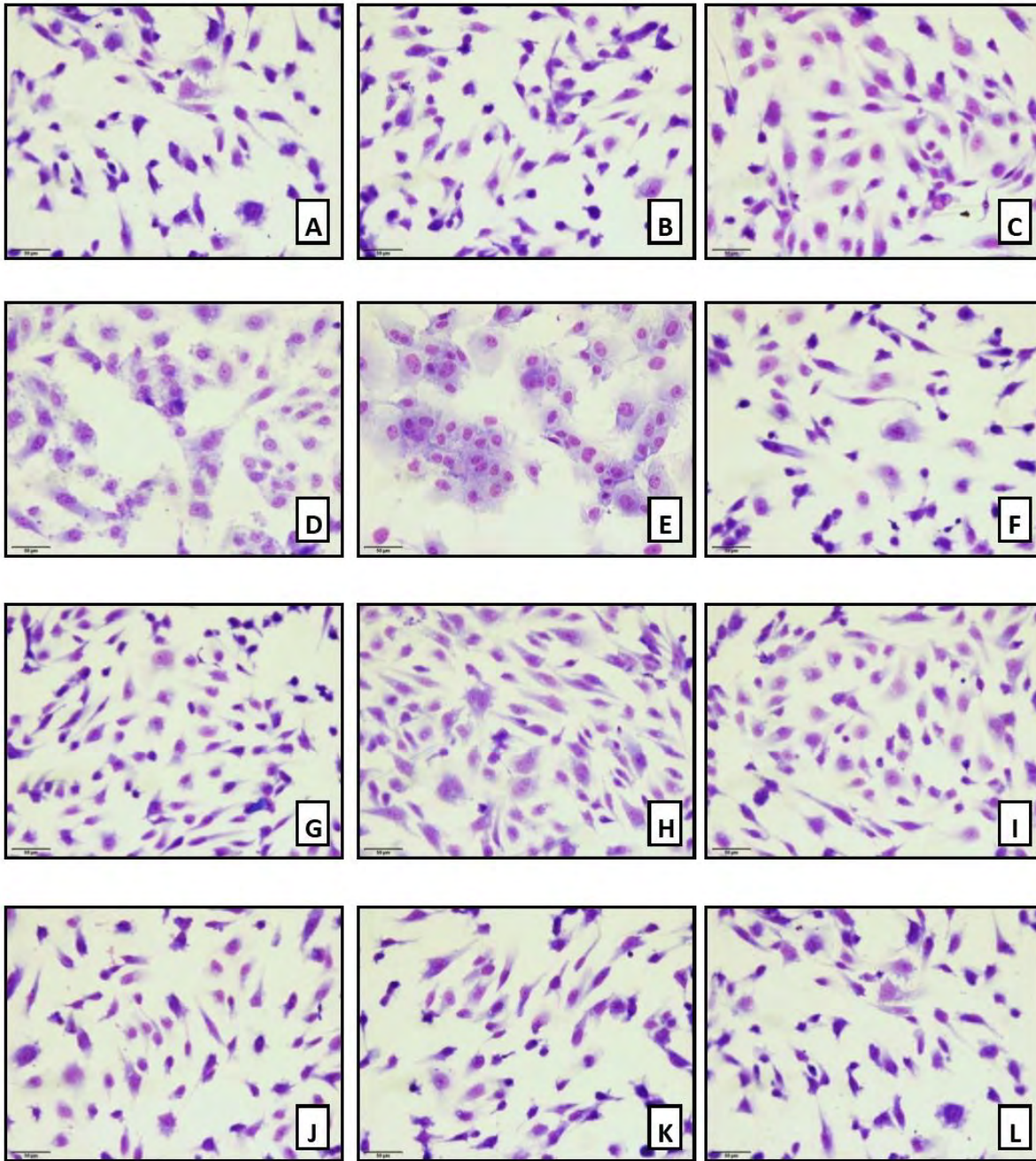


Figure S2: Photomicrographs (20X) of BMEC's treated with DEET (10 μ M) plus/minus metals (1 μ M). BMEC monocultures stained with Giemsa after treatments. A) Control; B) DMSO Control; C) DEET; D) DEET + Al; E) DEET + Fe; F) DEET + Ni; G) DEET + Sr; H) DEET + Co; I) DEET + Cu; J) DEET + Mn; K) DEET + Zn; L) DEET + DU. Magnification 20X, scale bar represents 50 μ m.

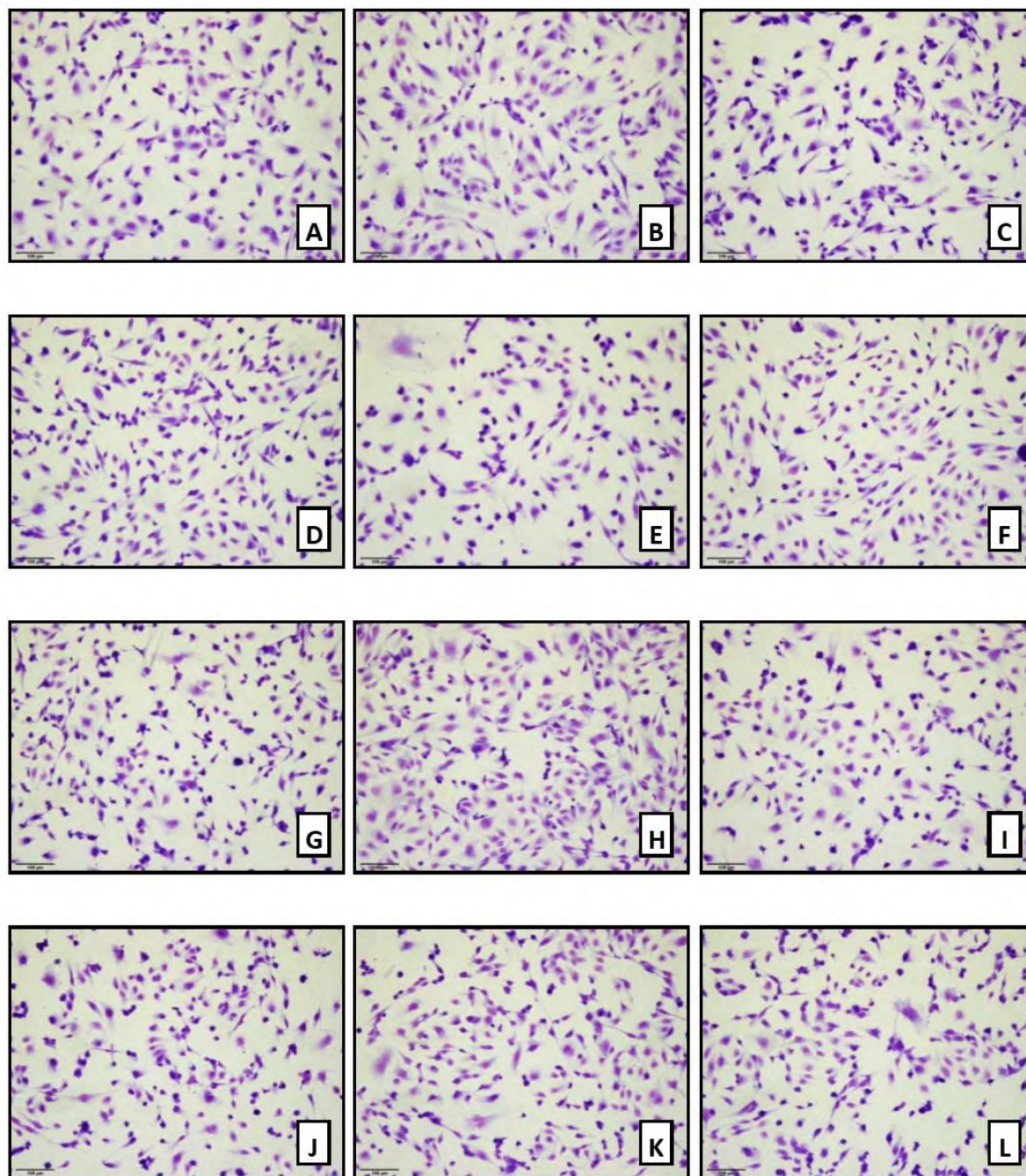


Figure S3: Photomicrographs (10X) of BMEC's treated with pyridostigmine bromide (PB, 10 μ M) plus/minus metals (1 μ M). BMEC monocultures stained with Giemsa after treatments. A) Control; B) DMSO Control; C) PB; D) PB + Al; E) PB + Fe; F) PB + Ni; G) PB + Sr; H) PB + Co; I) PB + Cu; J) PB + Mn; K) PB + Zn; L) PB + DU. Magnification 10X, scale bar represents 100 μ m.

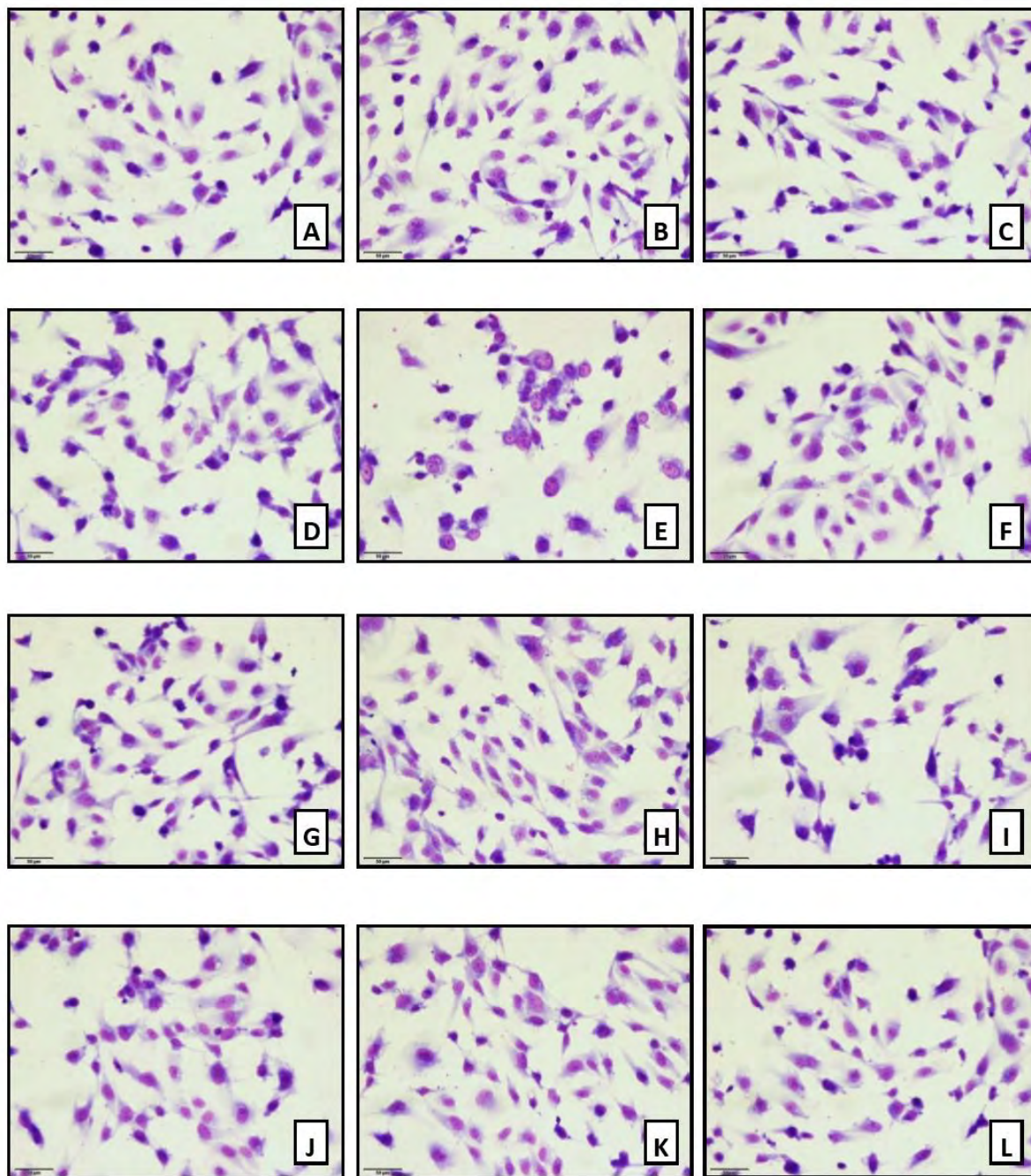


Figure S4: Photomicrographs (20X) of BMEC's treated with pyridostigmine bromide (PB, 10 μ M) plus/minus metals (1 μ M). BMEC monocultures stained with Giemsa after treatments. A) Control; B) DMSO Control; C) PB; D) PB + Al; E) PB + Fe; F) PB + Ni; G) PB + Sr; H) PB + Co; I) PB + Cu; J) PB + Mn; K) PB + Zn; L) PB + DU. Magnification 20X, scale bar represents 50 μ m.

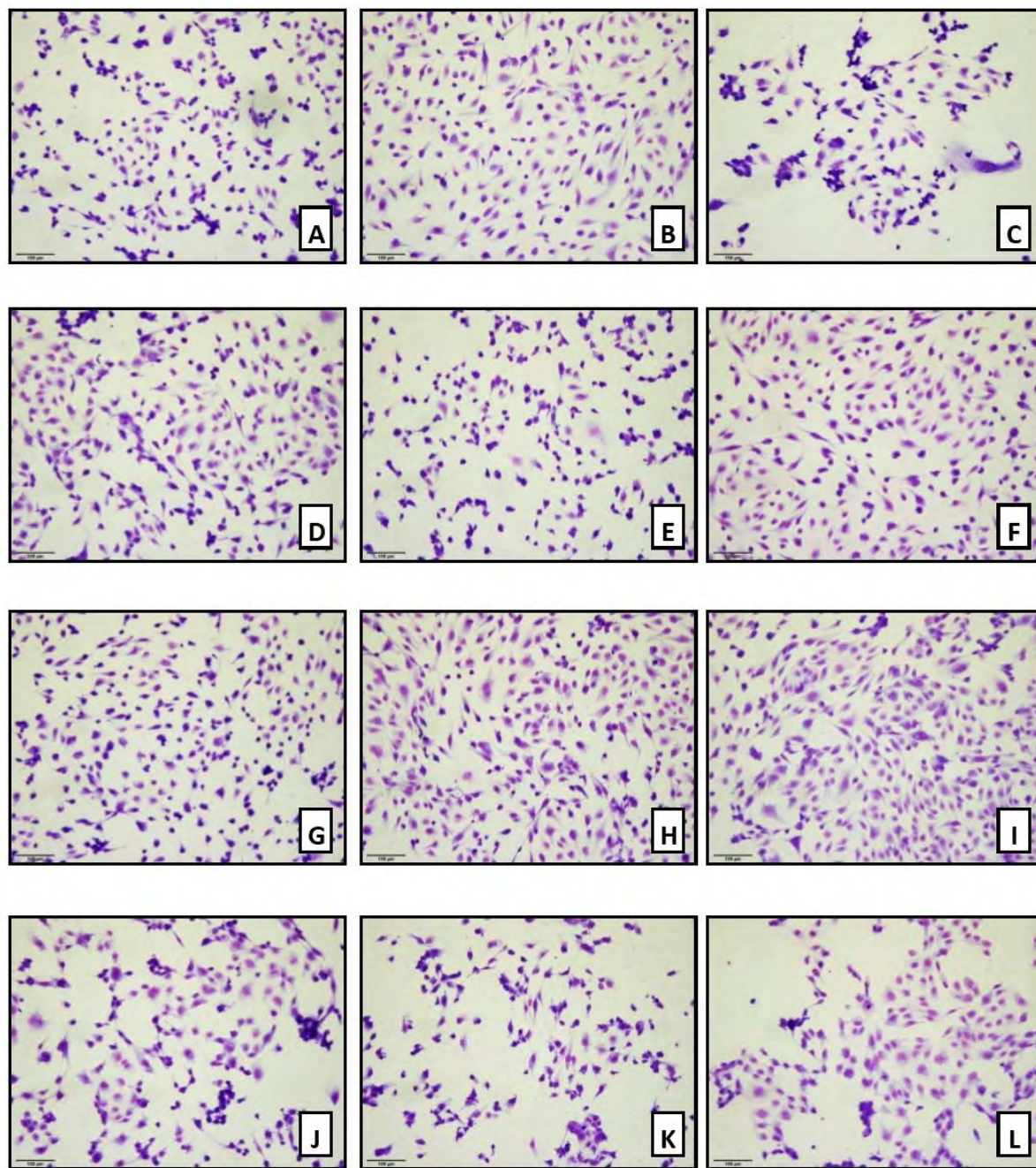


Figure S5: Photomicrographs (10X) of BMEC's treated with permethrin (PM, 10 μ M) plus/minus metals (1 μ M). BMEC monocultures stained with Giemsa after treatments. A) Control; B) DMSO Control; C) PM; D) PM + Al; E) PM + Fe; F) PM + Ni; G) PM + Sr; H) PM + Co; I) PM + Cu; J) PM + Mn; K) PM + Zn; L) PM + DU. Magnification 10X, scale bar represents 100 μ m.

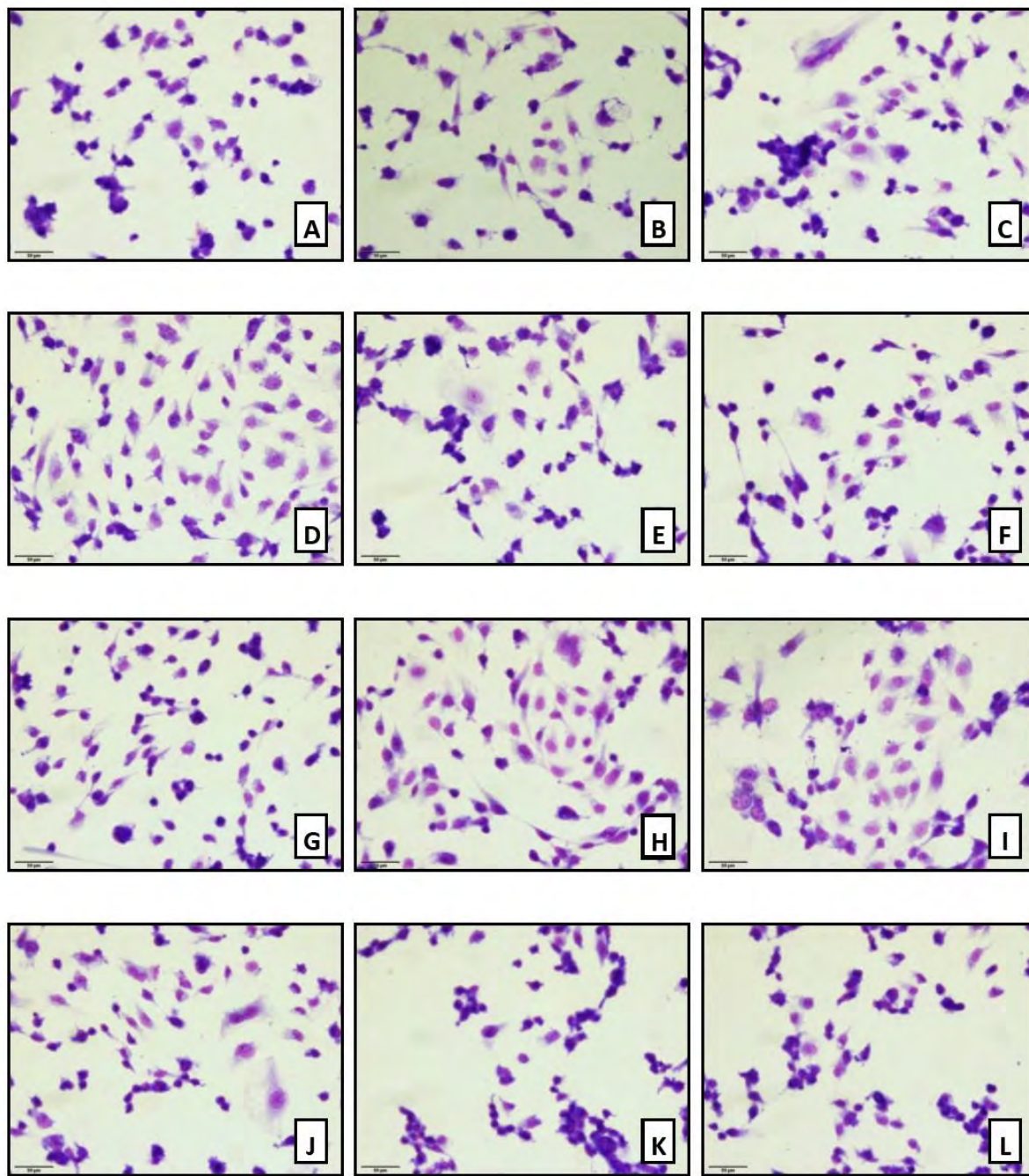


Figure S6: Photomicrographs (20X) of BMEC's treated with permethrin (PM, 10 μ M) plus/minus metals (1 μ M). BMEC monocultures stained with Giemsa after treatments. A) Control; B) DMSO Control; C) PM; D) PM + Al; E) PM + Fe; F) PM + Ni; G) PM + Sr; H) PM + Co; I) PM + Cu; J) PM + Mn; K) PM + Zn; L) PM + DU. Magnification 20X, scale bar represents 50 μ m.

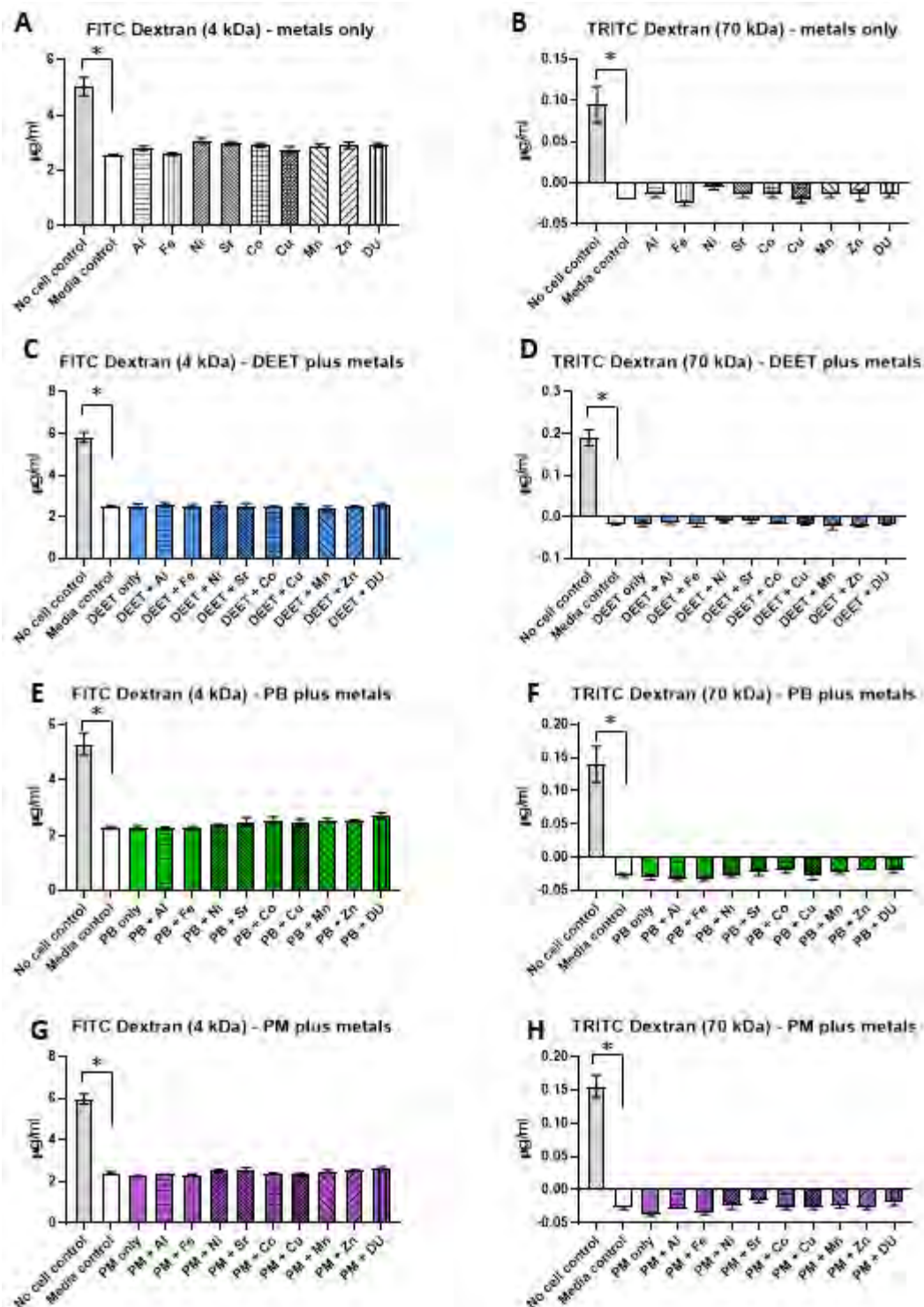


Figure S7: Transfer of fluorescent markers (4 kDa and 70 kDa) across a BBB transwell model after exposure to organic and metal compounds. Data presented as $\mu\text{g/ml}$ of FITC dextran (4 kDa) or TRITC dextran (70 kDa) that crossed over to the astrocyte side of the BBB model transwell after a known concentration presented to the endothelial side after exposure to organic and metal compounds. Gray bar indicates no-cell control wells. White bar represents media only control. Colored bars indicate organic treatment, pattern indicates subsequent metal treatment with no pattern indicating media only instead of metal. A) DEET (blue) plus metals, B) PB (green) plus metals, C) PM (purple) plus metals. * indicates significant p-value for comparison between groups indicated with connected lines.

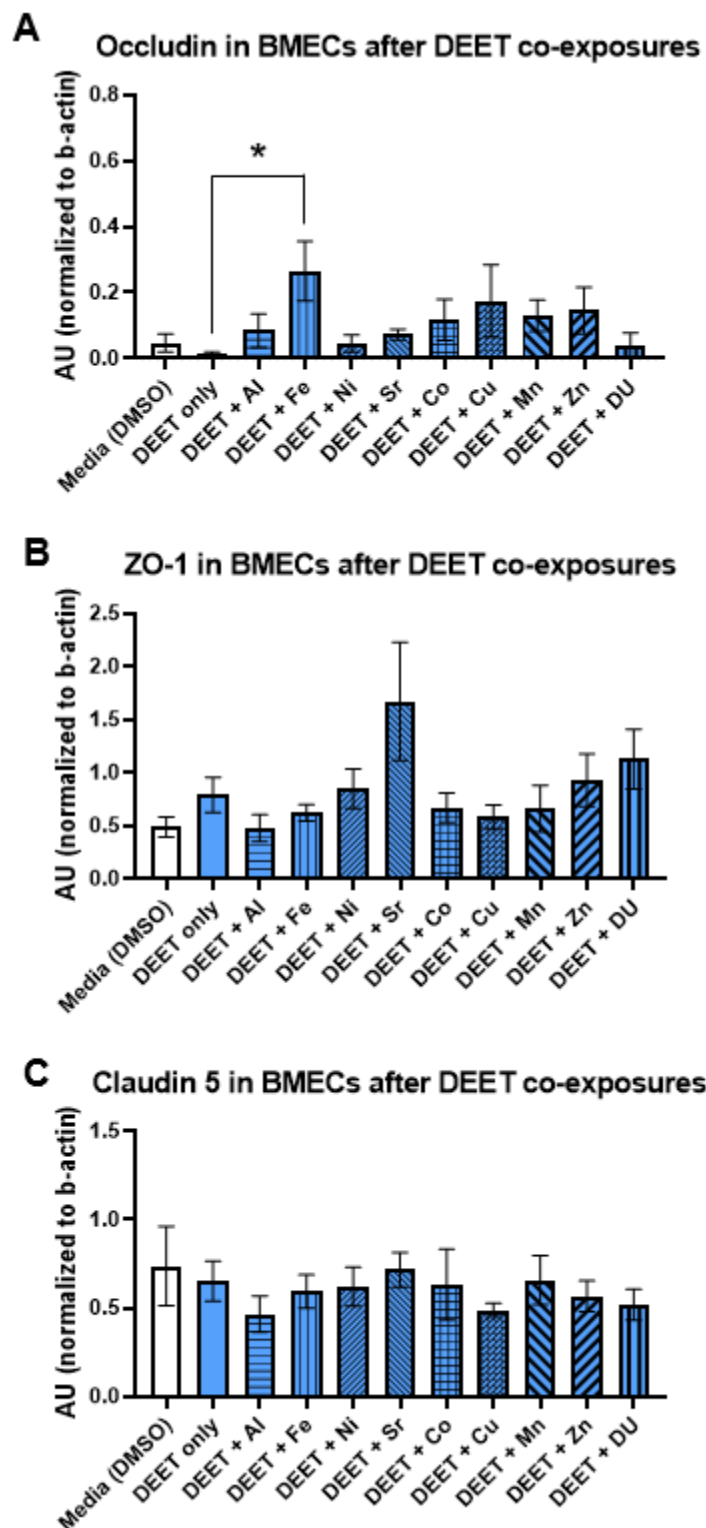


Figure S8: BBB protein expression in BMEC cultures after exposure to DEET and metal compounds. Data presented as arbitrary units of ratio of chemiluminescence value of target protein over within-subject value of β -actin. White bar represents media only control. Blue bars indicate DEET treatment, and pattern indicates subsequent metal treatment with no pattern indicating media only instead of metal. A) occludin expression, B) ZO-1 expression, C) claudin 5 expression. * indicates significant p-value for comparison between groups indicated with connected lines.

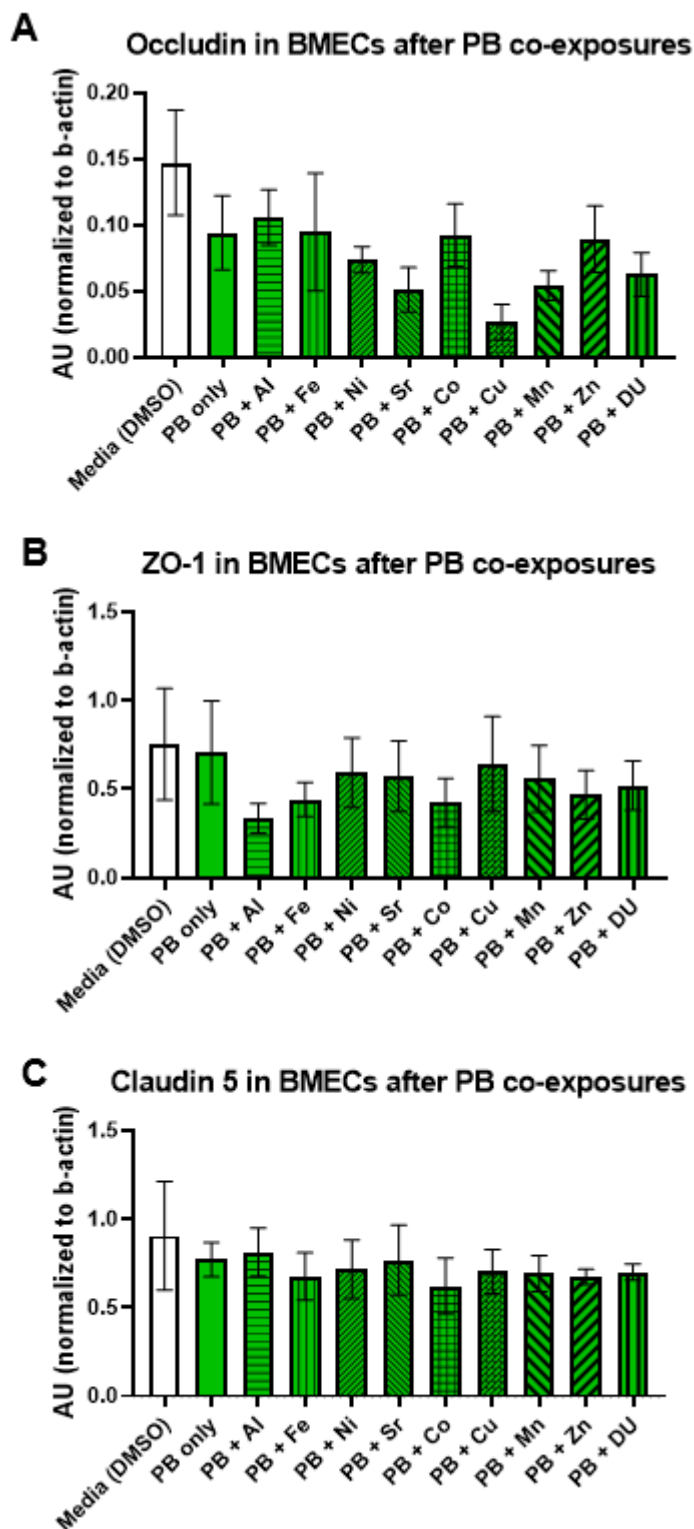


Figure S9: BBB protein expression in BMEC cultures after exposure to PB and metal compounds. Data presented as arbitrary units of ratio of chemiluminescence value of target protein over within-subject value of β -actin. White bar represents media only control. Green bars indicate PB treatment, and pattern indicates subsequent metal treatment with no pattern indicating media only instead of metal. A) occludin expression, B) ZO-1 expression, C) claudin 5 expression. No significant differences.

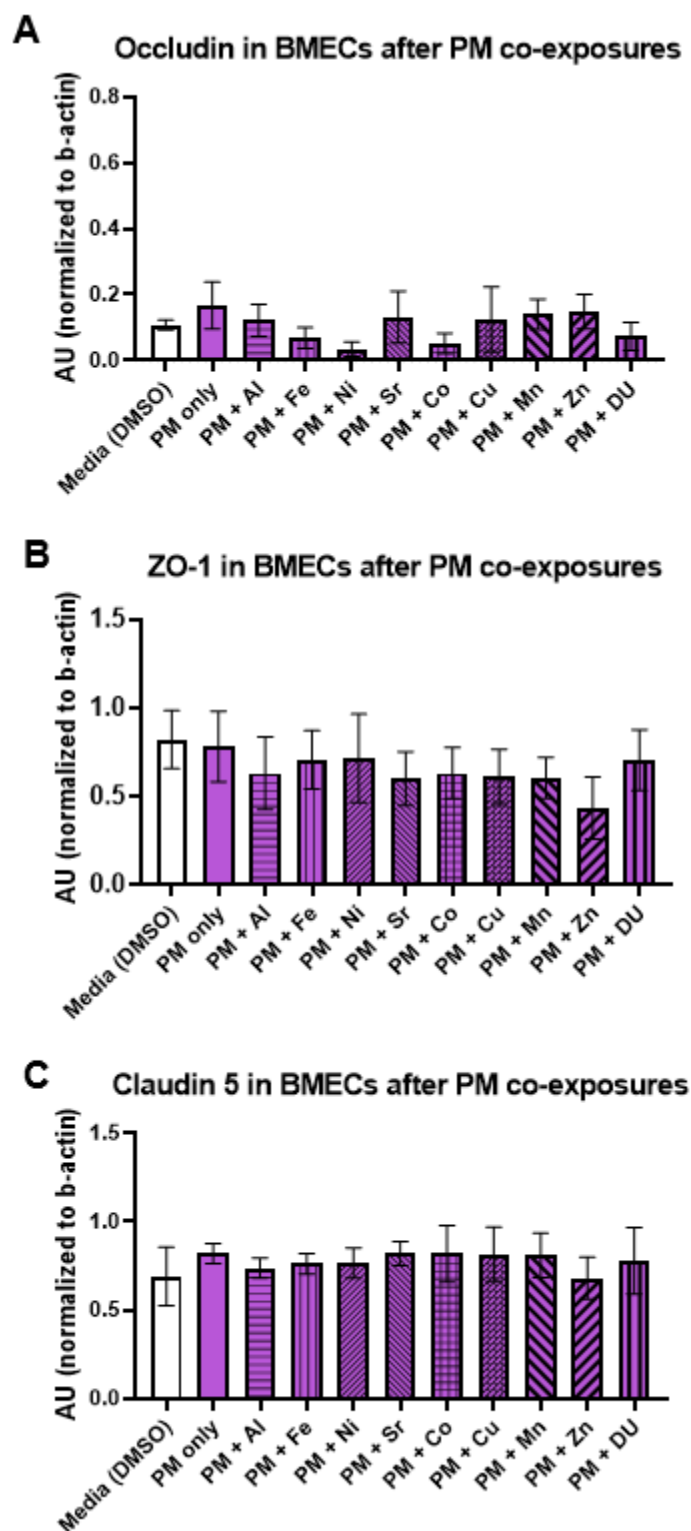


Figure S10: BBB protein expression in BMEC cultures after exposure to PM and metal compounds. Data presented as arbitrary units of ratio of chemiluminescence value of target protein over within-subject value of β -actin. White bar represents media only control. Purple bars indicate PM treatment, and pattern indicates subsequent metal treatment with no pattern indicating media only instead of metal. A) occludin expression, B) ZO-1 expression, C) claudin 5 expression. No significant differences.

ICP-MS operating conditions and parameters

Instrument Parameters

Nebulizer type	Concentric
Spray chamber	Conical, with impact bead
Sampler cone	Platinum, 1mm orifice diameter
Skimmer cone	Platinum, 0.7 mm orifice diameter
Sample uptake rate	1.0 ml/min
Sample read delay	45 sec

Plasma conditions

RF power	1400 W
Plasma argon gas flow	13.0 L/min
Auxiliary argon gas flow	0.80 L/min
Nebulizer gas flow	0.91 L/min

Mass spectrometer settings

Scanning mode	Peak jump
Sweeps	100
Dwell time	500 μ s
Channels/mass	1
Acquisition time	10 sec
Number of readings/replicate	3
Number of replicates	2

Table S1: ICP-MS operating conditions and parameters. Instrument parameters, plasma conditions, and mass spectrometer settings for the ICP-MS.

Antibody Table

Primary Antibodies					
Antibody	Host	Vendor, Cat #	Purpose	Conc.	Size (kDa)
Occludin (N-term)	Rb	ThermoFisher, 40-6100	BBB marker	1:50	63
ZO-1	Rb	ThermoFisher, 40-2200	BBB marker	1:50	63
Claudin-5 (4C3C2)	Ms	ThermoFisher, 35-2500	BBB marker	1:50	28
Beta-actin (13E5) x rab		CellSignaling, 4970S	Housekeeping (normalization)	1:10,000	48
Beta-actin (8H10D10) x mou		CellSignaling, 3700S	Housekeeping (normalization)	1:10,000	48

Table S2: Antibody information for protein expression analysis. Details of antibodies used in ProteinSimple Wes analyses.