4. Neurite Growth Research Project-JanPlan 2009

Investigating the effects of an antioxidant, Ginkgo Biloba, and an oxidative stressor, DEET, on neurite growth in fiddler crabs, *Uca pugliator*.

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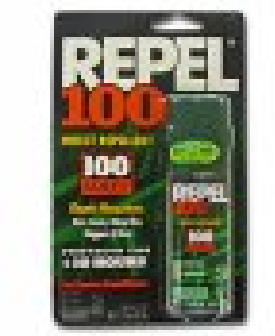
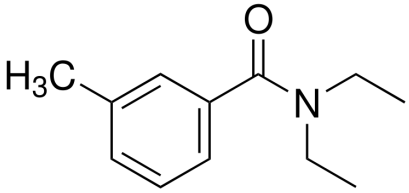
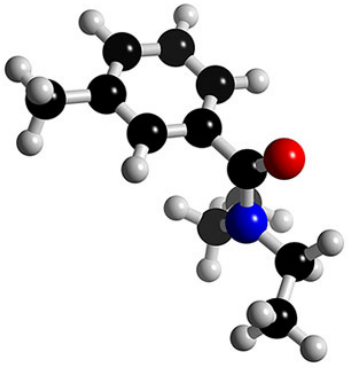
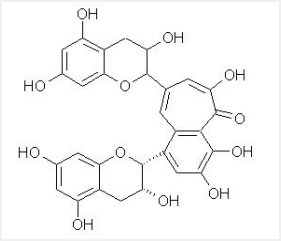


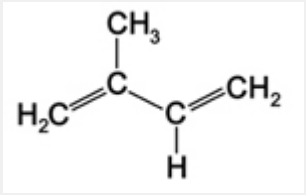
N,N-Diethyl-meta-toulamide. The active chemical in DEET:





Flavonoids and Terpenoids are the antioxidant elements in Ginkgo Biloba extract:





For many years the Tilden's research lab has focused on crustacean nervous systems, specifically the neural tissue of the fiddler crab, *Uca Pugilator.* Most of the resarch has focused on the effects of melatonin, a hormone, on neurite growth in neurons cultured from the X-organ/sinus gland complex in fiddler crabs. This particular study was designed with the idea that antioxidative additives may prolong/promote neurite growth and oxidative stressors may inhibit growth.

The fiddler crab was a great model organism for us in that the extraction of specific tissues from its eyestalk proved feasible in our undergraduate lab setting. Before delving into our project, Andrea briefed us on the structures we were about to examine.

Within the eyestalk sits an organ collectively known as the X-organ/sinus gland, analogous to the hypothalamus and pituitary gland in humans. The somata comprises the X-organ portion of the complex, while the synaptic endings (axons) constitute the sinus gland. The X-organ/sinus

gland regulates and secretes a number of hormones directly into the hemolymph, where it reaches target organs marked by hormone-specific receptors. Cardioactive hormone, pigment dispersing and concentrating hormones, crustacean hyperglycemic hormone, and molt inhibiting hormone, are some of the many integral hormones under the control of the X-organ/sinus gland.

The X-organ/sinus gland has three lobes, but we focused primarily on the tissue directly opposite the opalescent-blue sinus gland, which served as a visual landmark during dissection. We harvested only the X-organ cells because they most closely resemble human hippocampus cells, which are most important for making synaptic connections during learning and memory.

In researching common dietary and herbal supplements used in Alzheimer's patients, we came across the antioxidant Ginkgo Biloba extract. Ginkgo Biloba extract has been used in Chinese medicine for centuries to treat nearly every type of ailment, and has relatively recently made it to the United States as a potential treatment and/or preventative measure for Alzheimer's and other neurodegenerative conditions. The flavonoid compounds found in the leaves of Ginkgo Biloba are antioxidants, which bind to free radicals in the cells. By binding to the free radicals, the flavonoids prevent the free radicals from "stealing" electrons from the growing neurite membranes. We hypothesized that this antioxidant property would result in enhanced neurite growth. Furthermore, Ginkgo Biloba extract contains terpenoid compounds, which are less studied, but are thought to enhance neural connections.

We needed a way to induce oxidative stress within the growing neurites. In past studies hydrogen peroxide was used but we decided to treat the neurons with a commonly used insect repellant, DEET. We chose Repel 100, which is 100% DEET (N,N-Diethyl-meta-toulamide). We hypothesized that the free radicals found in the highly toxic substance would inhibit neurite growth and therefore decrease the overall amount of neurite growth in the cells treated with this compound.

The purpose of our project was to determine the impact of an oxidative stressor, DEET, and an exogenous antioxidant, Ginkgo Biloba, on neurite growth in neurons extracted from the X-organ of the fiddler crabs *Uca pugilator*.

After removing the eyestalks from anesthetized crabs, we microdissected the eyestalks and removed neural tissue from the X-organ. We enzymatically digested the tissue fragments using trypsin, separated the tissue to break loose individual neurons, and cultured the cells for a period of forty-eight hours.

Neural cells grown in a normal culture medium were used as controls. Our experimental cells were cultured in a medium treated with either 100% DEET insect repellent (at a 50% lethal dose), or with 24% standardized Ginkgo Biloba extract (10X the human dose).

After forty-eight hours, the cell cultures were examined under a brightfield microscope at 640X magnification in a glass-bottom culture dish and photographed using AxioVision software. Neurite areas and cell body areas were calculated using Image J, and a ratio of neurite to cell body was calculated.

Our results do not fully support our hypothesis. Both of our DEET and Gingko Biloba additives resulted in a significantly different neurite growth than our controls (Figure 1). It was expected that the Gingko Biloba additives would have a higher neurite growth than the control due to its chemical ability to prevent the free radicals from "stealing" electrons from the growing neurite membranes. However, DEET, a potentially toxic substance, had more neurite growth than Gingko Biloba, a health supplement. DEET and Ginkgo Biloba exhibit antagonistic properties of oxidation and antioxidation, respectively, and yet both led to a strong and significant stimulatory response. This could mean that the oxidative influences of DEET are stimulatory and not inhibitory, which does not support our hypothesis. The reasons for the increased neurite growth of DEET is unknown however there is a very strong possibility that DEET simply increases neurite growth. Further studies are needed to discover the underlying mechanisms. However, in other studies it was found that acetylcholinesterase (AChE), the enzyme that catalyzes the reaction for the neurtransmitter acetylcholine (ACh) to be broken down into choline and acetic acid, enhances neurite growth and synapse development. This enzyme also functions to return an stimulated neuron to its resting state by inhibiting ACh. Hence, if AChE activity is suppressed by an inhibitor such as a pesticide like DEET, the concentration of ACh increases in the synapse causing over stimulation of neurons. This suggests that a potent dose of DEET could over stimulate neurons to a certain toxicity. Another study found that animals treated with DEET alone produced significant increases in cortical ACh receptor binding. In contrast, what we do know at this time is that DEET does indeed stimulate neuron growth, at least under the perimeters of this experiment. Therefore, there might be some undiscovered mechanism within the fiddler crabs that instead of inhibiting neurite growth it stimulates it. This experiment is highly worthy of further study due to the fact that other oxidative stressors, such as hydrogen peroxide, was found by previous Tilden studies to inhibit neurite growth. Additionally, because both additives are made of a mixture of different chemicals, there could be many different unknown stimulating/inhibiting factors, which is why more research is needed on this subject.

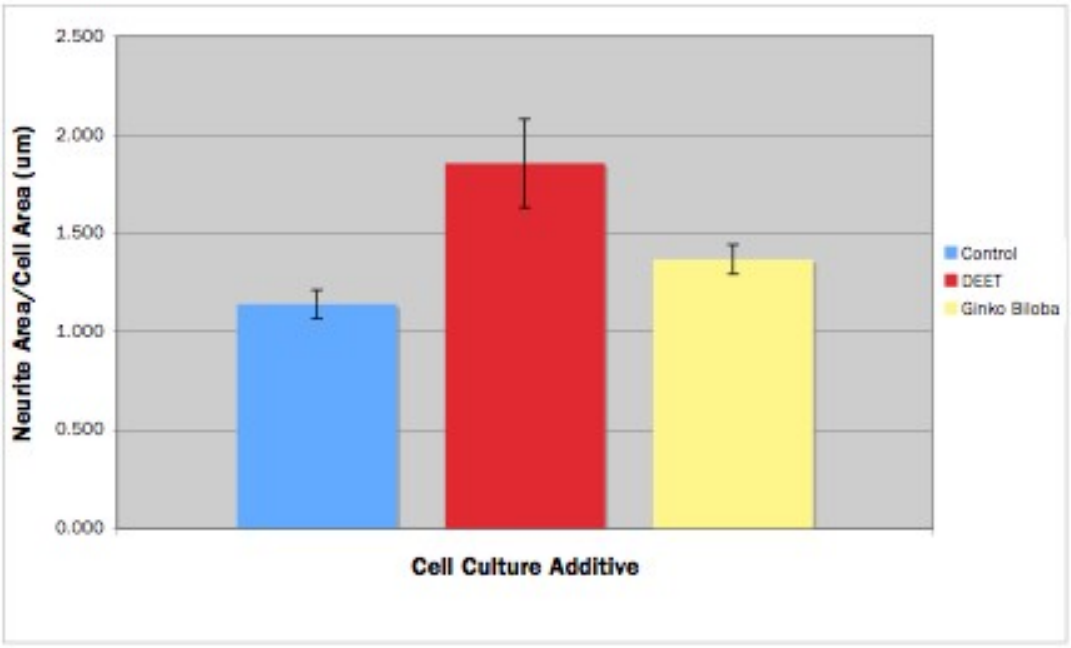


Figure 1. Neurite growth in neurons extracted from X-organs of *Uca pugliator* as a function of cell culture additives. Controls were treated with normal culture medium, n=100; DEET n=79 and Ginko Biloba n=158.

We believe that some other, more methodological variables for these results could be that Gingko Biloba cultures accumulated bacteria from various unknown sources, possibly diminishing and/or aiding neurite growth in cultures. Another variable was the possible contaminants entering culture dishes during an allotted waiting period of 48 hours could have altered growth. Additionally, the DEET sample size is much smaller than the Gingko sample size. Our data was compiled over a two-week span, and our sample sizes ranged from n=79 for DEET to n=158 for Gingko Biloba.

If we had had more time for this experiment we would have liked to examine the effects of treating neurons previously exposed to the DEET compound, and therefore already predisposed to oxidative stress, with the Ginkgo Biloba extract. Further research could be done on not only the enhancement properties, but also the repairative properties of Ginkgo Biloba extract. We also would liked to have examined a larger sample size of cells treated with DEET, as this was our lowest sample size. We also encountered some problems with bacterial infections, most likely stemming from an infected culture medium or trypsin solution. We still got results despite the bacteria growth in some of our cultures, but with more time we would have eliminated the infections and hopefully examined more cell growth.

We would like to thank the Colby College Biology Department for their lovely facilities. We also thank Andrea Tilden, our wise professor, for her wonderful guidance and support. Additionally, we are grateful for the incredible teaching assistant abilities of Escar Kusema and Max Mutter, and Ruth Langton and Jen Myers for their helpful insight.

DEET technical fact sheet from the National Pesticide Information Center:
http://npic.orst.edu/factsheets/DEETtech.pdf

This experiment was conducted by Ben Rooney, Victor Gagne, Amy Campbell, Christina Mok and Kimberly Parker.