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14. ABSTRACT

In a rat model of Gulf War Illness (GWI) we tested the hypothesis that mitochondrial dysfunction in central to GWI pathology and that treatment with dichloroacetate (DCA) will be beneficial. GWI was induced by exposure to a combination of chemicals used during the Gulf war to protect personnel (pyridostigmine bromide, permethrin, DEET) daily for a period of one month. DCA treatment was performed 5 months post-exposure and lasted 1 month. Thus far we tested 16 male rats (GWI, GWI-DCA, control, and control-DCA, n=4). Results from this small number of animals suggest that GWI rats have increased anxiety-like behavior however, no effect of DCA on anxiety was detected. GWI-DCA rats appeared to regain the memory loss detected in GWI rats in the Morris water maze test. Densitometric analysis of coronal brain sections immunostained with antibodies to Iba1 showed that DCA treatment decrease the expression of Iba1, a marker of microglia activation, in the dentate gyrus of GWI down to the levels detected in control rats. These results imply that the animal model we use is appropriate to test our hypothesis and that DCA may have beneficial effects on GWI-related behavior and pathology.

15. SUBJECT TERMS
Gulf War Illness, acetylcholinesterase inhibitors, insecticides, stress, dichloroacetate, mitochondrial dysfunction, oxidative stress, fatigue, inflammation, memory, anxiety. neuromuscular pain, pyruvate dehydrogenate.

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1. INTRODUCTION:

This is a pre-clinical trial to study the potential therapeutic value of dichloroacetate (DCA) for the treatment of GWI. DCA is an experimental drug that has been safely used for decades to treat rare congenital mitochondrial diseases. DCA is a well-known inhibitor of pyruvate dehydrogenase kinase and, thus, keeps the pyruvate dehydrogenate complex in its active unphosphorylated state, which enhances the function of mitochondria by increasing the oxidative phosphorylation and the production of adenosine triphosphate (ATP) while decreasing the level of lactic acid. The rationale for targeting mitochondria is based on recent studies in GWI patients and animal models of the disease that have indicated an involvement of mitochondrial dysfunction along with closely-related oxidative stress and inflammation. While investigating DCA as a therapy for GWI, the project is designed to test, in a well characterized rat model of GWI, the hypothesis that 1) mitochondrial dysfunction is central to the pathology of GWI, 2) exercise challenge exacerbates mitochondrial dysfunction in GWI, and 3) oral treatment with DCA will improve brain function and behavior.

2. KEYWORDS:

Gulf War Illness, acetylcholinesterase inhibitors, insecticides, stress, dichloroacetate, mitochondrial dysfunction, , oxidative stress, chronic fatigue, inflammation, memory, anxiety, neuromuscular pain, pyruvate dehydrogenate,

3. ACCOMPLISHMENTS:

o What were the major goals of the project?

The major goals of the project are:

- Approval by local IRB/IACUC and ACURO
- **Major Task 1:** Exposing the rats to PB/PET/DEET and restraint stress daily for a period of one month in order to develop a GWI-like disease in rats.
- **Major Task 2:** Therapeutic treatment with DCA with or without additional stress exposure, in vivo studies (behavior tests) and tissue harvesting
- **Major Task 3:** Analysis of blood samples
- **Major Task 4:** Analysis of brain samples by biochemical, immunohistochemical, and ^{1}HMRS techniques
- **Major Task 5:** Analysis of videos and data collected from the behavior analysis of elevated plus maze and Morris water maze

During the first year of the grant the goal was 1) to get approved by the local committees and ACURO and 2) to expose rats to chemicals and stress to develop GWI-rats for later treat them with DCA to see the therapeutic effect of the drug on behavior and pathology (Major Task 1 and 2).

After receiving the final approval by ACURO in December 2019, we proceeded to order the first cohort of 16 male 3-month-old Sprague-Dawley rats from Charles River and continued with the 1 month exposure to chemicals and stress (**Major Task 1**). Our plan was to be ordering a new cohort of 16 rats every month after the exposure phase while waiting for the disease to develop. Due to the outbreak of the COVID 19 pandemic in March 2020, our facility restricted all new animal work and animal orders. However, we were able to continue our work on the rats that had already been exposed. After waiting 5 months for the disease to get established on these rats, we proceeded to treat them with DCA, performed the behavioral tests (fatigue tests, elevated plus maze test, and Morris water maze test) and tissue harvesting (**Major Task 2**). We have finished analyzing the videos and data collected from the behavior tests **(Major Task 5**) and started the process of analyzing the collected tissue (brain, blood, soleus muscle, and spinal cord) (**Major Task 3 and 4**).

As soon as the restriction on ordering animals was lifted at the end of August 2020 we ordered a second cohort of rats. These animals have already been exposed to PB/PET/DEET and restraint stress daily for a period of one

month (**Major Task 1).** We are now waiting for the disease to develop before we will proceed to treat them with DCA (**Major Task 2**).

A third cohort of rats has already been ordered and are in the process of being exposed to PB/PET/DEET and restraint stress (**Major Task 1**).

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

Sixteen male Sprague-Dawley rats 3 months of age were ordered from Charles River. Eight rats (4 cages) were randomly assigned to model the Gulf War Illness (GWI) while the other 8 rats (4 cages) were assigned to the control group. The rats in the GWI group were exposed orally to 1.3 mg/kg pyridostigmine bromide (PB) dissolved in the drinking water and topically to 0.13 mg/kg permethrin (PER) and 40 mg/kg DEET dissolved in 70% ethanol applied to a shaved area on their back. After each daily topical chemical exposure, rats were placed in a Plexiglas rat restrainer for 15 minutes (restraint stress). Rats received exposure to the chemicals and stress daily for 1 month. Control (unexposed) rats were similarly given a dermal application of 70% ethanol (the PER and DEET diluent). Rats' weight was monitored weekly during the 1 month exposure and periodically throughout their entire lifespan. At 9 months of age the therapeutic phase started. For that phase, 4 of the 8 rats in the GWI group and 4 of the 8 rats in the control group were randomly assigned to be treated with dichloroacetate (DCA) for 1 month while the other 4 GWI and 4 control rats were assigned to remain untreated. Before treatment started and again after the 1 month of DCA treatment all the rats were given a fatigue test consisting in running on an accelerating treadmill (starting at 10 m/min and increasing 1 m/min every 2 min until behavioral criteria was met for fatigue). During the last week of treatment, rat's anxiety and memory were evaluated by the elevated plus maze test and the Morris water maze test, respectively. After testing was completed, rats were euthanized and their brains, lumbar spinal cord, soleus muscles, and blood were harvested for analysis.

The results presented below are based on the small number of rats studied so far and thus no statistical analysis have been performed yet. As the number of rats will increase and new results will be generated, they will be added to the current data set and statistical analysis will be performed. Since the current number of exposed rats (GWI rats) treated with DCA is so small any attempt to comment on the potential effect of DCA treatment is purely suggestive.

Results: As shown in Figure 1, no apparent differences in body weight were detected between the GWI and control rats during the 1 month exposure nor during the subsequent 5 months during which the disease developed. Although rats appeared to tolerate well the oral treatment with DCA and no noticeable behavioral differences between DCA-treated and untreated rats were observed, we detected a 10% decrease in body weight in animals treated with DCA (both in GWI rats and control rats). Food intake by DCA-treated rats was 25% lower than that of untreated rats. Before DCA treatment started and again after it ended 1 month later, all rats were given two fatigue tests (on two consecutive days) consisting in running on an accelerating treadmill. Results from the fatigue test, show that GWI-rats, either DCA-treated or untreated, stayed longer running on the treadmill than control rats during the two tests performed after the treatment period (Figure 2). The reason for this unexpected outcome is not clear; however, we observed that while most of the GWI rats were docile and accepted the challenge of running, control rats were more uncooperative making it more difficult to encourage them to run. Results from the elevated plus maze test (Figure 3) show that GWI rats spent less time in the opened arms than control rats, a sign of increased anxiety-like behavior. Interestingly, GWI rats traveled longer distances and at greater speeds than control rats throughout the 3 min duration of the plus maze test. In the Morris water maze test, all the rats showed after 4 days of training that they learned the task of finding the submerge platform (Figure 4 left panel). Probe trials performed 1h and 24h after the last training trial on day 4 showed that untreated GWI rats spent less time than GWI-DCA-treated rats and control rats 0in the quadrant where the platform used to be (Figure 4 middle and right panel). The difference between untreated GWI rats and the ones treated with DCA increased in the 24h probe trial suggesting that GWI rats have decreased

memory function and that DCA is able to restore it. Right after testing rats were euthanized and brains, lumbar spinal cord, soleus muscles, and blood were harvested for analysis. The cryoprotected right hemibrains have already been sectioned into a series of 10 sets with 50 µm-thick sections. Two sets of sequential sections spanning the entire hemibrain had been immunostained with antibodies against Iba1 to visualize microglia and against GFAP, a marker of reactive astrocytes. Sections of the hippocampal dentate gyrus immunostained with Iba1 are shown in Figure 5. Although we are currently performing a more in-depth analysis of the Iba1 immunostained sections, results from the densitometric analysis suggest that DCA treatment restores the level of Iba1 expression down to the level detected in control rats.

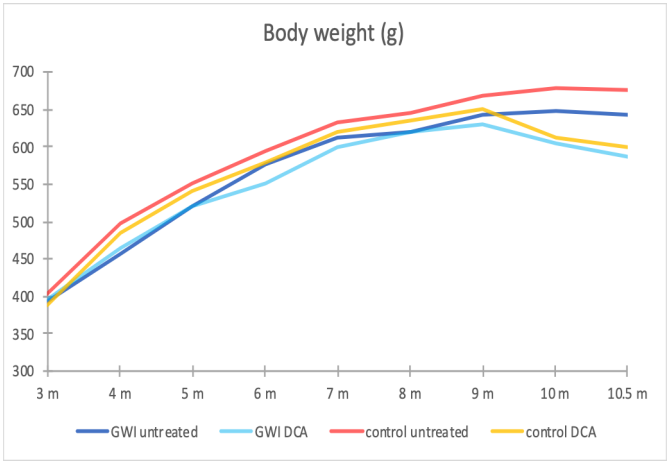

Figure 1. Rats' body weight

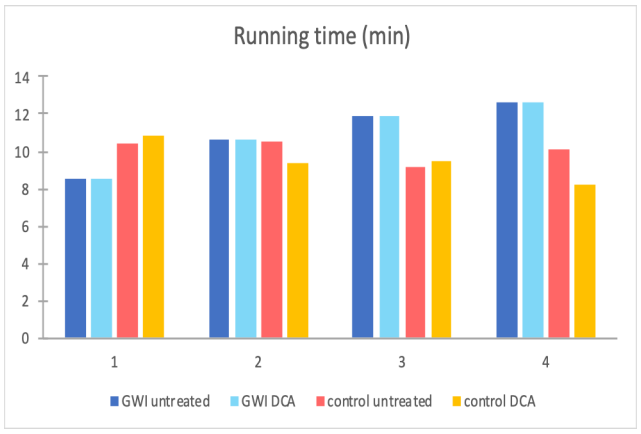

Figure 2. Fatigue tests 1 and 2 performed before the treatment period and 3 and 4 performed after the treatment period.

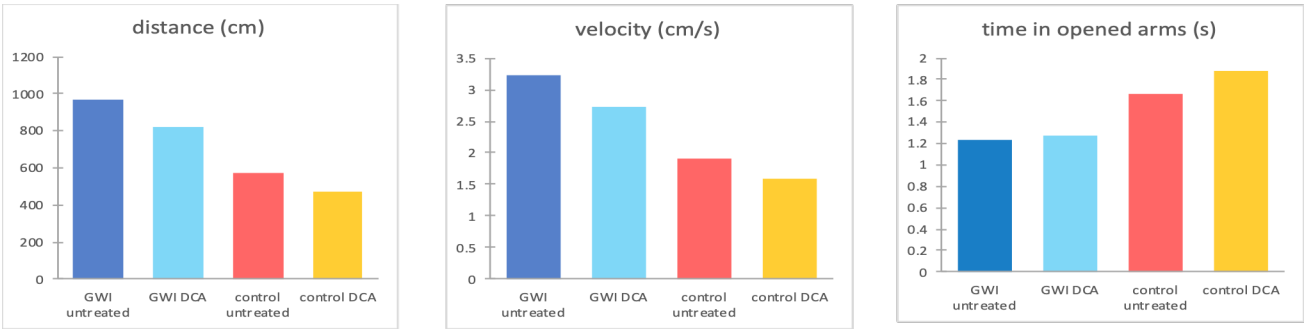

Figure 3. Elevated plus maze test

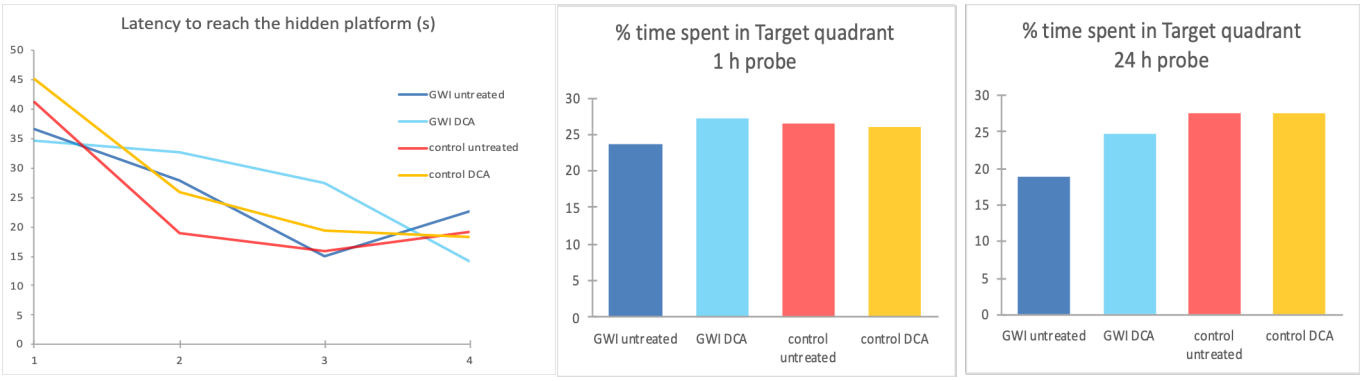

Figure 4. Learning and memory in the Morris water maze test

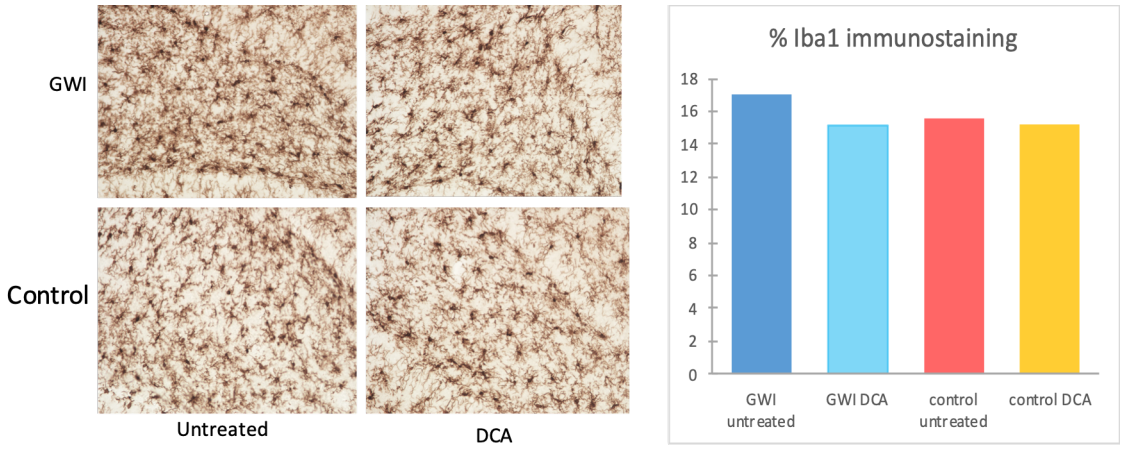

Figure 5. Iba1 immunostaining of microglia in the dentate gyrus. Magnification 200x

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

Nothing to report.

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

Nothing to report.

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

During the next reporting period, we plan to finish the analysis of all the tissue collected in the first cohort of rats and continue with exposing and treating the next planned cohorts of male rats. As we complete the treatment, behavioral tests, and tissue collection for each rat cohort, the collected tissue and behavior data will be analyzed and will be compiled to the existing results. We plan to start reporting results as soon as all the experiments on male rat cohorts are completed. Similar to the planned schedule for males, we expect to start experiments in female rats during the next reporting period.

4. IMPACT:

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

Nothing to report.

- **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**

We observed that some of the rats, particularly rats in the control group that had not been exposed to chemicals nor restraint stress, refuse to run on the treadmill during the fatigue test. We believe that the refusal to run was due to a rat's attitude of non-cooperation rather than a sign of pain or physical dysfunction because it occurred immediately during the onset of each fatigue test rather than after a period of exertion. To improve the rats' motivation to run, we will increase the period for habituation to the treadmill. In addition, we will add the rotarod test. The rotarod test is widely use in rodents to measure motor function. It may be a better test to unveil neuromuscular fatigue or pain in GWI rats. Before performing the rotarod test, an amendment to the ACORP protocol will have to be approved by the IACUC and from the awarding agency Grants Officer.

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

There has been an actual delay on the progression of the project due to the COVID 19 pandemic and the temporary ban on ordering animals. We are planning to resolve this delay by increasing the number of animals that we include in each subsequent cohort. As personnel gets more acquainted with all the procedures involved in the project, we do not foresee any problem in increasing the number of rats we work on in each cohort.

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

Decreased expending during the reporting period is due to the temporary halt in ordering animals.

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

There have been no significant changes in use or care of animals or in use of biohazards and/or select agents.

6. **PRODUCTS:**

- **Publications, conference papers, and presentations**

Nothing to report.

7. **COLLABORATING ORGANIZATIONS**

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

Name:	Isabel Carreras
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	0000-0002-7120-6749
Nearest person month worked:	4
Contribution to Project:	Dr. Carreras exposed the rats to PB/PET/DEET and restraint stress and monitored the oral treatment with DCA. She monitored rat's body weight

	Together with Dr. Tognoni performed the fatigue test, euthanasia and tissue collection.
Funding Support:	-
Name:	Alpaslan Dedeoglu
Project Role:	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0003-1156-0874
Nearest person month worked:	0.6
Contribution to Project:	Dr. Dedeoglu has been overseen all aspects of the project, and provide advice.
Funding Support:	-
Name:	Christina Tognoni
Project Role:	Post-doctoral fellow
Researcher Identifier (e.g. ORCID ID):	0000-0001-5073-8693
Nearest person month worked:	1
Contribution to Project:	Dr. Tognoni performed behavioral testing. Together with Dr. Carreras performed the fatigue test, euthanasia and tissue collection.
Funding Support:	VA
Name:	Jonathan Lopez
Project Role:	Lab Technician
Researcher Identifier (e.g. ORCID ID):	0000-0002-5196-004X
Nearest person month worked:	1
Contribution to Project:	Mr. Lopez, cryoprotected, sectioned and immunostained bemibrain sections.
Funding Support:	VA

○ **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

Nothing to report.

ADDITIONAL NOTES:

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