HIGH SCHOOL EDITION
Journal of Student Research
Volume 12 Issue 3 (2023)

Establishing Nitric Oxide Levels as a Biomarker for Fibromyalgia Diagnosis and Rheumatoid Arthritis Diagnosis

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ABSTRACT

Fibromyalgia (FM) is a chronic, hyperalgesic disorder leaving musculoskeletal pain throughout the body without tissue damage, ultimately affecting about 1 in 25 to 1 in 50 people in the United States. Although prevalent, its diagnosis is complicated and long due to its ambiguity of official signs of its presence. Patients wait an estimated 2.3 years and see about 3.7 physicians on average before being diagnosed with this disorder. To combat the lack of secure physiological ways to determine fibromyalgia in patients, measuring nitric oxide (NO) levels in the body appears to be a promising biomarker. Nitric oxide often works as a mediator of localized inflammation, although in fibromyalgia, a non-inflammatory disorder, NO levels are predicted to be heightened in the blood due to a lack of arginase, a competitive inhibitor of NO production. *Drosophila melanogaster* were used to model this disease due to its available homologous gene TRPA1, a notorious pain receptor. A "mimicking" fibromyalgia type was developed to ensure that the results were specific to physiologically accurate forms of fibromyalgia and not just other disorders that present similarly. Results showed that the fibromyalgia model had significantly higher levels of nitrite and nitrate in the body serum than its mimicking type, even though the organisms behaved similarly when exposed to painful stimuli. Even so, arginase was also significantly higher than the mimicking group. Regardless, measuring derivatives of nitric oxide appears to be a promising first step in finding a biomarker that will pinpoint fibromyalgia upon diagnosis.

Background

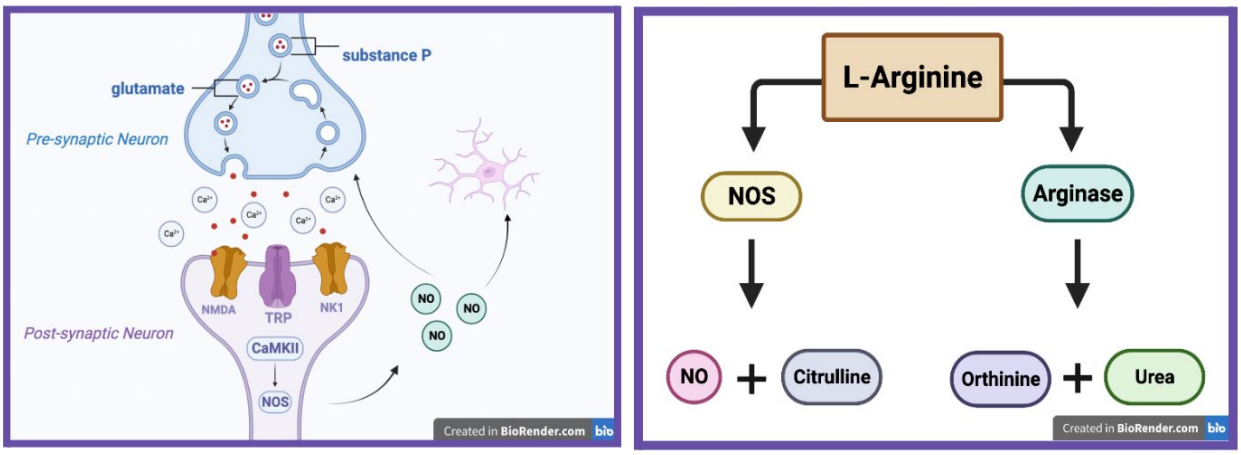
Fibromyalgia (FM) is a chronic, nociceptive disorder classified as a central sensitization syndrome in which pain arrives almost spontaneously, is exaggerated, and prolongs the response by a nociceptor. This unfortunate condition affects around 1 in 25 to 1 in 50 people in the United States and runs rampant among women—roughly 7.7% of women and 4.9% of men in the U.S. are diagnosed with fibromyalgia (Vincent, A. et. al). The diagnosis of FM is complicated because it is almost "invisible" and physicians must rule out other causes of chronic pain in the body such as rheumatoid arthritis (RA), hypothyroidism, and systemic lupus erythematosus (SLE). Oftentimes, physicians use the American College of Rheumatology preliminary diagnostic criteria which involves the fulfillment of a widespread pain index, symptom severity score, generalized pain in at least 4 regions, the presence of symptoms for over 3 months, and the exclusion of all other causes of the aforementioned criteria. In recent years, scientists have developed the FM/a blood test in order to make diagnosis more accurate and timely (American Family Physician n.d.). The test works by analyzing white blood cells for abnormal chemokine and cytokine patterns because fibromyalgia isn't inflammatory, so it will have differing cytokines than other related disorders. Unfortunately, the test is reported to have a false positive rate of 29% and 31% for patients with SLE and RA, respectively (Straub, L. et. al). Additionally, the test has not shown whether it can distinguish between those with Fibromyalgia and those without in patients that have not been diagnosed yet but present with typical Fibromyalgia symptoms. However, nitric oxide (NO) levels in the exhaled

breath might be a useful tool in making a clearer diagnosis of fibromyalgia mimicking disorders to help close that false-positive gap.

Normally, pain sensation occurs through afferent C nerve fibers sending out excitatory neurotransmitters such as glutamate and substance P into the synaptic cleft. When this occurs, NK1 and AMPA receptors are activated and allow for the transmission of these neurotransmitters to the postsynaptic neuron. In fibromyalgia, the membranes of postsynaptic neurons are hyperexcitable, so they allow for an influx of calcium into the postsynaptic membrane. Calcium ions then bind to calmodulin in the calcium/calmodulin-dependent protein kinase II. This protein is key in causing long-term potentiation of signals, leading to hyperalgesia in fibromyalgia. This kinase phosphorylates NO synthases with L-arginine to produce citrulline and NO (Jones, R. et. al). This relationship directly contributes to NO going back to create a loop: penetrating the neuronal membrane to activate soluble guanylyl cyclase, which in turn, forms cGMP as a second messenger. cGMP modifies the activity of calcium channels and protein kinases to amplify pain signals. On the other hand, NO independent of cGMP can (a) activate surrounding glia and hyper excite the presynaptic membrane to produce more stressors or (b) combine with cysteine thiol from protein residues to form S-nitrosothiol (SNO). Going off of option A, NO works by activating glutamate in the presynaptic neuron to be released and again be taken up by AMPA and NMDA—restarting the cycle. For option B, S-nitrosylation is the process in which TRP channels are activated, specifically TRPV1 and TRPA1 (Nishida, M. et. al). TRP cation channels, or transient receptor potential channels, are often activated by stimuli like temperature, light intensity, and touch, but their high Ca^{2+} permeability and reaction to NO makes them susceptible to activation when there is calcium overload. For instance, TRPA1 (ankyrin 1), a subtype of TRP channels, is activated by extreme temperature, acidity, and mechanical pressure. In fibromyalgia, this channel experiences calcium overload because of NO's cyclical mechanisms which causes hypersensitivity to the previously mentioned stimuli. Although fibromyalgia's etiology is vague, it is most popularly linked with anxiety, depression, and childhood trauma causing hypermethylation of the promoter region of TRPA1 which causes pain thresholds to be lowered, allowing for overactivation (Achenbach, J. et. al).

Currently, there is a test done to evaluate the effectiveness of asthma treatment via detection of elevated levels of NO in the breath of patients exhaling for ten seconds. The hypothesis is that overexpression of inducible NO synthase leads to increased NO and indirectly activates eosinophilic inflammation in the lungs, the main characteristic of asthmatic patients. The same goes for fibromyalgia: increased NO. Because nitric oxide production is directly linked to arginase activity, measuring these two factors looks to be a promising tool in diagnosis.

To model "mimicking" fibromyalgia, flies are treated with reserpine which is a drug that is typically used to treat high blood pressure, however it can slow down the central nervous system and induce a depression-like phenotype in model organisms.



(A) **(B)**

Figure 1A. The main pathway in which painful stimulus is transmitted throughout the nervous system.
Figure 1B. A simple breakdown of the competition between NOS and Arginase.

Statement of Purpose

The purpose of this research project is to determine a physiological marker that can characterize the diagnosis of Fibromyalgia more accurately than today's technology.

Research Question

Are concentrations of nitric oxide significantly higher in fibromyalgia patients' blood and exhaled breath than those without the disorder?

Hypotheses

1. If *Drosophila melanogaster* is genetically mutated to overexpress the TRPA1 gene to model fibromyalgia, then they will have higher concentrations of nitrite and nitrate in their hemolymph serum than both the control group and the mimicking fibromyalgia group that was treated with reserpine.
2. If *Drosophila melanogaster* are genetically mutated to overexpress the TRPA1 gene to model fibromyalgia, then they will have less arginase activity in their hemolymph serum than both the control group and the mimicking fibromyalgia group.
3. If *Drosophila melanogaster* are genetically mutated to overexpress the TRPA1 gene to model fibromyalgia, then they will release more nitric oxide in their "breath" than both the control group and the mimicking fibromyalgia group.
4. If *Drosophila melanogaster* are genetically mutated to overexpress the TRPA1 gene to model fibromyalgia and a group of wild type flies are treated with reserpine to induce a mimicking fibromyalgia type, then the two groups will behave similarly but have significantly different levels of nitrite/nitrate in their tissue.

Materials and Methods

- TRPA1 GAL-4 flies - Bloomington Stock Center #27593
- TRPA1 UAS flies - Bloomington Stock Center #26264
- NOS GAL-4 flies - Bloomington Stock Center #24283
- NOS UAS flies - Bloomington Stock Center #56829
- PAD4 CNS driver - Professor William Ja, FAU
- PAD4 ubiquitous driver - Professor William Ja, FAU
- wild-type flies
- reserpine-treated wild-type flies (mocks symptoms of chronic pain)
    - Reserpine powder (99%) - Sigma Aldrich
- 2 x Nitrite/Nitrate Assay kit - Cayman Chemical #780001
- 3D-printed custom collection cap (Figure 2)
- $FeSO_4 \cdot 7H_2O$
- 125-micron nylon filter mesh
- 1 ¼" diameter x 4" height plastic fly vials
- 4.31 (2.041 g) Touch Test Sensory Evaluator - North Coast Medical and Rehabilitation Products
- 9% Acetic Acid solution
- Stopwatch
- Glass homogenizer
- Sodium Nitrite
- Formula 4-24 Instant Drosophila Medium, Blue

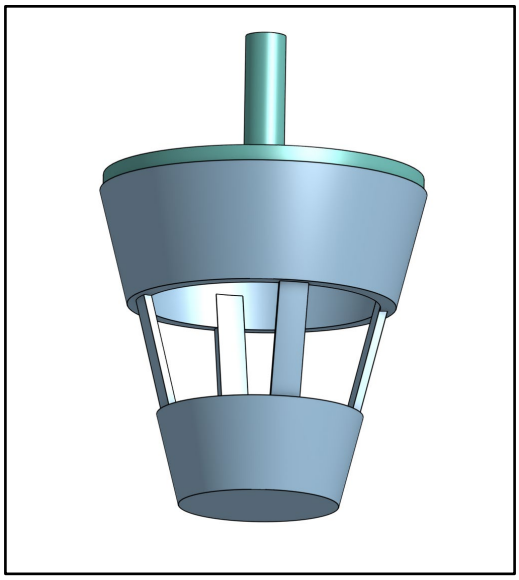


Figure 2. The 3D design used to create the collection cap in OnShape.

Genetic Crosses of GAL4/UAS flies

1. After receiving the flies, they are kept at a 12 hour day/night cycle at room temperature.
2. Once populations have grown to at least 50 flies per mutant, begin crossing.
3. Separate the males and females of each of the mutant flies. Males are typically smaller in body size than females. Females typically have larger, rounder, and white abdomens whereas males have thin, black abdomens.
4. Put opposite sexes of separate mutants that modify the same gene into vials with sufficient food (i.e. TRPA1 GAL-4 males are crossed with TRPA1 UAS virgin females).
5. To know if the crosses were successful, the following phenotypes should be observed:
    a. TRPA1 GAL4/UAS: white-apricot eyes (orange)
    b. NOS GAL4/UAS: white eyes

Reserpine treatment

1. Prepare a liter of Nutri-agar according to the directions on the packet.
2. Once boiling and homogenous, weigh out 0.0304 grams of reserpine powder on the digital scale.
3. Dispense the reserpine powder into the nutri-agar and mix thoroughly.
4. Before the mixture can harden, pour about 15 mL into each vial.
5. Clean up the sides (if needed) and cap each of them with a cotton plug.
6. Store in the fridge at 2-8ºc until ready to use.

Ferrous Sulfate Solution ($FeSO_4 \cdot 7\ H_2O$)

1. Measure out 25 grams of Iron (II) sulfate using a digital scale.
2. Add the Iron (II) sulfate to 500 ml of distilled water in a 1 L glass beaker.
3. Dissolve solution
4. Pour solution into a closed glass container for storage at room temperature away from direct light.

Acetic Acid Solution (9%)

1. Measure 26.2 ml of 6M Acetic acid in a graduated cylinder.
2. Add to 180 ml of distilled water.
3. Swirl until homogenous.
4. Store in a closed, glass container at room temperature and away from direct light.

Whole-tissue Nitrate/Nitrite Assay

1. Place 20 flies into the bottom of a glass homogenizer and add in 1.5 ml of 1x PBS solution.
2. Using the glass pestle, grind up flies until homogenous in the solution.
3. Pour the sample into 2.5 ml centrifuge tubes and centrifuge at 10,000 x g for 20 minutes.
4. Optional: Ultracentrifuge the supernatant solution at 100,000 x g for 30 minutes.
5. Prepare the reagent.
    a. Assay Buffer: Dilute assay buffer vial to 100 ml with MilliQ water. Store buffer at 4ºC when not in use.
    b. Nitrate Reductase Enzyme: Add 1.2 ml of assay buffer to vial and keep on ice during use. Store at -20ºC when not in use.
    c. Nitrate Reductase Cofactor: Add 1.2 ml of assay buffer to vial and keep on ice during use. Store at -20ºC when not in use. Store at -20ºC when not in use.
    d. Nitrate Standard: Remove the vial stopper slowly and add in 1 ml of assay buffer. Vortex so that all powder is in the solution. Store at 4ºC when not in use.
6. Follow the procedure as described in the Nitrate/Nitrite Colorimetric assay kit (Cayman Chemical).
7. Read the absorbance at 540 nm or 550 nm with a plate reader.

Whole-tissue Arginase Assay

1. Place 20 flies into the bottom of a glass homogenizer and add in 1.5 ml of 1x PBS solution.
2. Using the glass pestle, grind up flies until homogenous in the solution.
3. Pour the sample into 2.5 ml centrifuge tubes and centrifuge at 10,000 x g for 20 minutes.
4. Optional: Ultracentrifuge the supernatant solution at 100,000 x g for 30 minutes.
5. Bring all reagents to room temperature prior to assay, preheat the arginine buffer to 37ºC.
6. Urea Standard Preparation:
    a. Mix 24 µl 50 mg/dL urea and 176 µl of water.
    b. Add 50 µl mM Urea Standard 50 µl dH_2O to separate wells of 96-well plate.
7. Arginase Reaction:
    a. Combine 4 vol of Arginine buffer and 1 vol of Mn solution.
    b. Add 40 µl of each sample to 2 separate wells. Add 10 µl of the previously made solution (step 7, part a) into one of the two sample wells.
    c. Incubate plate for 2 hours at 37ºC.
8. Urea Reagent:
    a. Combine equal volumes of Reagent A and Reagent B.
    b. Add 200 µl of combined reagents to all wells. Tap the plate to mix. Incubate for one hour at room temperature
9. Place in plate reader at optical density of 430 nm.

"Exhaled" Nitric Oxide Assay

1. Collection caps:
    a. Using hot glue, adhere small trapezoidal cut-outs (roughly the size of the "windows" of the collection caps) to the supports of the cap on the outside.
    b. Repeat part (a) until all windows are closed off by mesh on the outside.
2. Add food and 40 flies into each of the 1 ¼" diameter x 4" height plastic fly vials.
3. Fit the collection caps to sit two-thirds of the way into the vials so that none of the windows should be over the rim of the vials.
4. Pipette 80 µl of ferrous sulfate solution into the basins of each of the caps. Close off with either a foam plug or the custom printed lid.
5. Allow flies and solution to incubate for 48-72 hours.
6. Collect samples of ferrous sulfate solutions into 2.5 ml centrifuge tubes and centrifuge at 10,000 x g for 20 minutes.
7. Optional: Ultracentrifuge the supernatant solution at 100,000 x g for 30 minutes.
8. Prepare the reagent.
    a. Assay Buffer: Dilute assay buffer vial to 100 ml with MilliQ water. Store buffer at 4ºC when not in use.
    b. Nitrate Reductase Enzyme: Add 1.2 ml of assay buffer to vial and keep on ice during use. Store at -20ºC when not in use.
    c. Nitrate Reductase Cofactor: Add 1.2 ml of assay buffer to vial and keep on ice during use. Store at -20ºC when not in use. Store at -20ºC when not in use.
    d. Nitrate Standard: Remove the vial stopper slowly and add in 1 ml of assay buffer. Vortex so that all powder is in the solution. Store at 4ºC when not in use.
9. Follow the procedure as described in the Nitrate/Nitrite Colorimetric assay kit (Cayman Chemical).
10. Read the absorbance at 540 nm or 550 nm with a plate reader.

Mechanical Withdrawal Assay

1. Preparation of the Larvae
    a. Take 15 (3rd instar) out at once and wash them in PBS wash.
    b. Dry off the larvae when you pick them up one at a time to perform the assay.
2. Performing the assay
    a. Calibrate the microscope and place a clean dish under it.
    b. Using the tweezers, place a dry larva onto the dish into the desired spot.
    c. Next, using the desired filament, gently touch the larva ten times (whether or not a response is elicited).

NOTE: A "response" is denoted as a 360º roll away from the stimulus or repeated jerking movements, thrashing of the body and tail due to the stimulus.

Chemical Withdrawal Assay

1. Preparation of the Larvae
    a. Take 15 (3rd instar) out at once and wash them in PBS wash.
    b. Dry off the larvae when you pick them up one at a time to perform the assay.
2. Performing the assay
    a. Calibrate the microscope and place a clean dish under it.
    b. Using the tweezers, place a dry larva onto the dish into the desired spot.
    c. Pipette 3 µl of 9% Acetic acid solution onto the bodies of larvae and observe if a response occurs within a 20 second time frame.

NOTE: A "response" is denoted as a 360º roll away from the stimulus or repeated jerking movements, thrashing of the body and tail due to the stimulus.

Results

Chemical Withdrawal Assay

This assay was used to determine if there is a heightened sensitivity among the fibromyalgia and reserpine groups. Each group was tested in 10 different trials with 15 flies per trial.

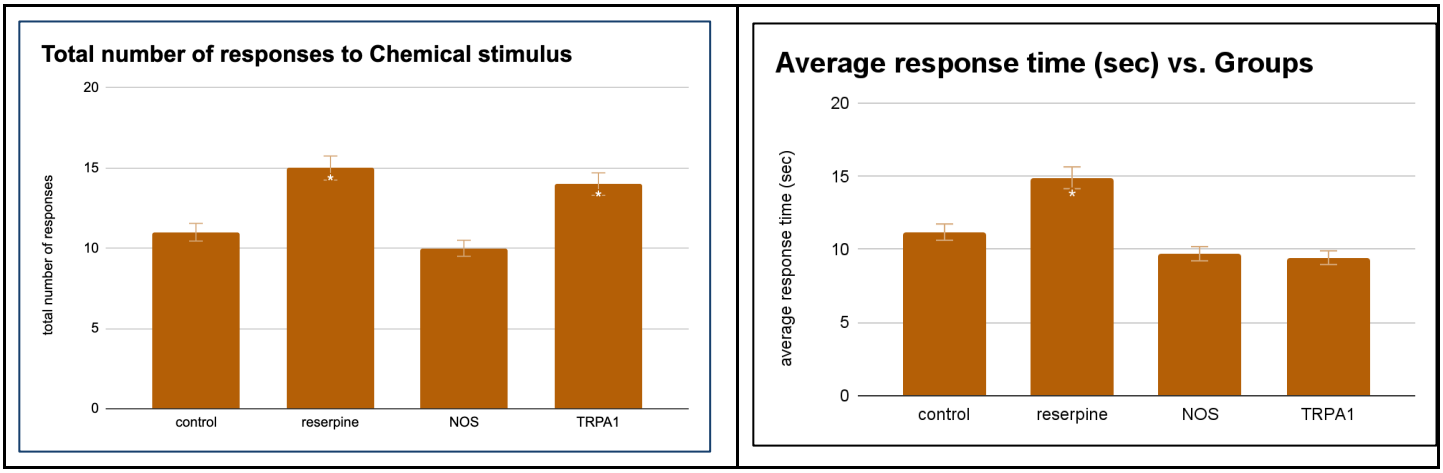


Figure 3. The Chemical Withdrawal assay shows the number of larvae that responded to being saturated in 9% acetic acid and how quickly they would react. TRPA1 mutant larvae reacted equally as often as reserpine-treated larvae, however, TRPA1 mutants reacted to stimulus 5.47 seconds faster on average. (T-test, $p<0.05$ between reserpine vs NOS, reserpine vs control, reserpine vs TRPA1 for response time). There were no other significant differences in response time for the rest of the T-tests, however the TRPA1 group reacted to stimulus 40.0% more often than the NOS group and reacted 27.3% more often than the control group.

Mechanical Withdrawal Assay

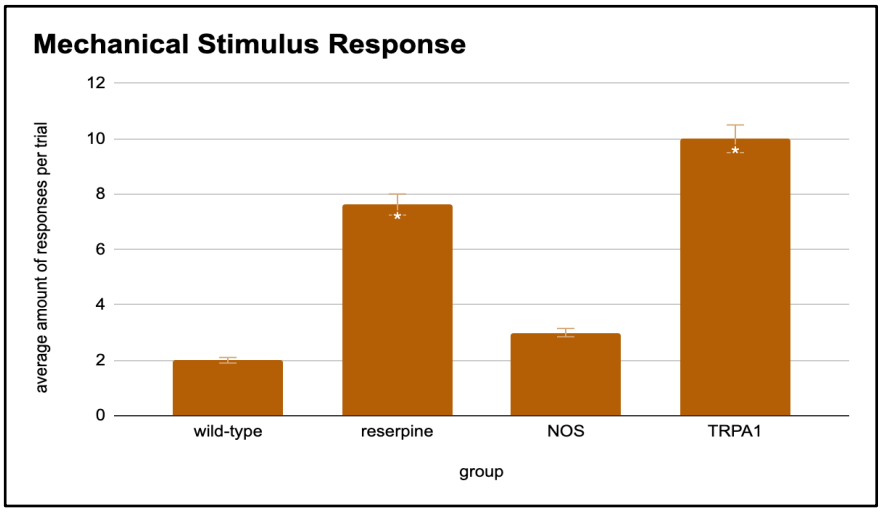


Figure 4. The Mechanical Withdrawal assay assessed each groups' sensitivity to touch and their ability to respond to it by avoidance which was observed as a roll away from the stimulus. All T-tests were significant between each of the groups ($P<0.05$).

Whole-Tissue Nitrate/Nitrite Assay

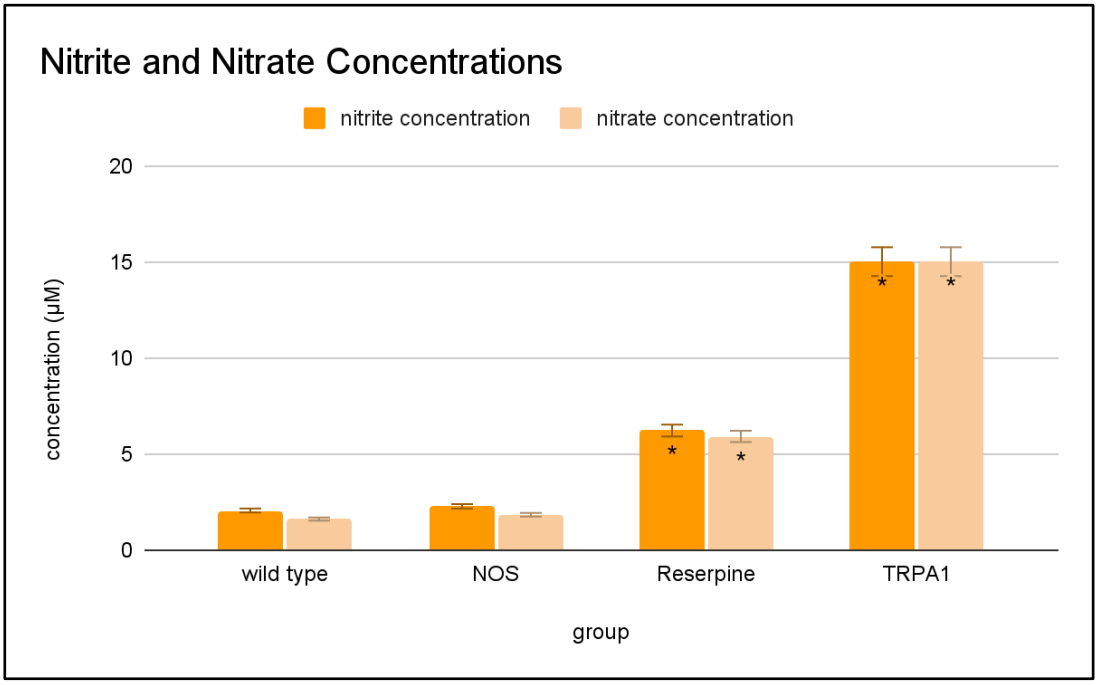


Figure 5. Nitrate/Nitrite concentration was quantified using absorbance of light at 540 nm. The TRPA1 group absorbed significantly more than any of the other groups in T-tests ($P<0.05$).

Arginase Activity Assay

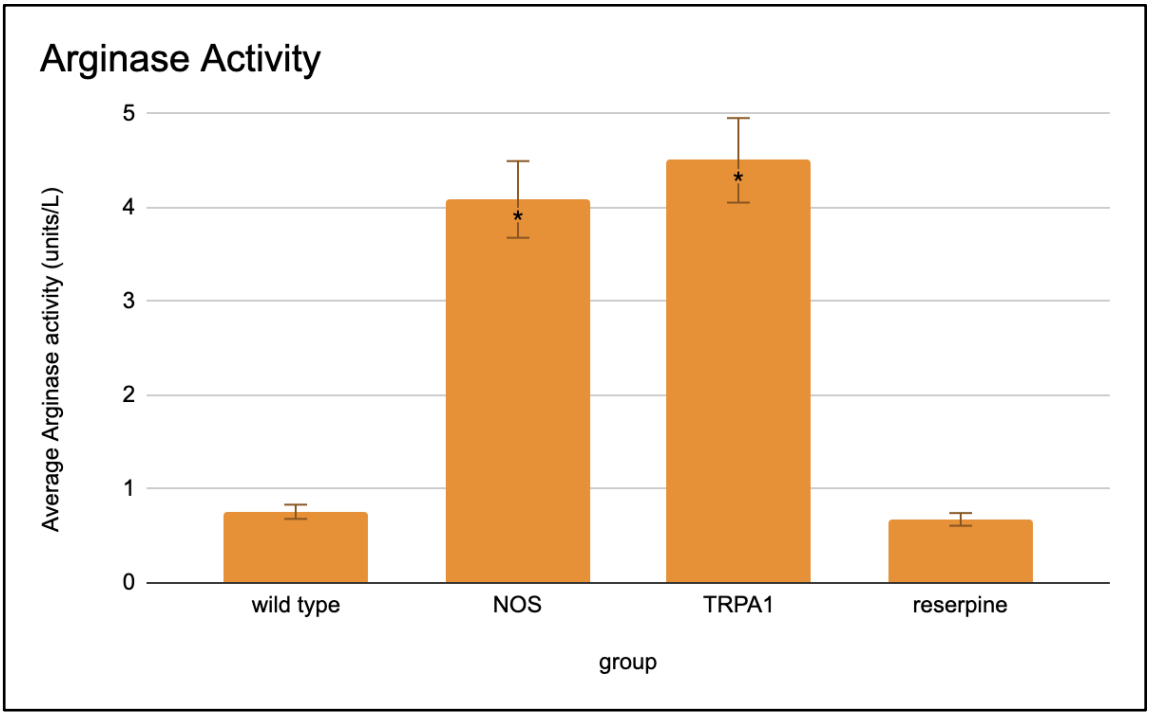


Figure 6. The Arginase activity assay was used to assess the activity of nitric oxide synthase's competitor, arginase, to understand the relationship between the two. All comparisons upon T-test were significant ($P<0.05$) except for NOS vs TRPA1 and NOS vs reserpine ($P>0.05$).

Discussion

The first hypothesis was supported by the nitrite/nitrate concentration assay. Because nitrite (NO_2) and nitrate (NO_3) are based upon the same basic components in nitric oxide, testing for them in the hemolymph was able to elucidate the relationship between fibromyalgia presence, its mimicking conditions as modeled by reserpine, and the absence of it on nitric oxide levels in the body. Although the reserpine-treated group did have higher concentrations of nitric oxide than the control group, the TRPA1 group modeling fibromyalgia genetically exhibited concentrations about 7 times higher than the reserpine group. Thus, measuring nitric oxide levels in serum samples makes a strong and simple distinction between what can and cannot be classified as fibromyalgia. The second hypothesis was not supported by arginase activity assay. Arginase and nitric oxide synthase are competitive inhibitors of each other, therefore it was predicted that the NOS and TRPA1 groups would have significantly lower activity than either of the two other groups because they were thought to have the highest concentration of nitric oxide, the byproduct of the competitive inhibitor. However the results may be explained by arginase being produced in greater concentrations in response to the increased concentrations of nitric oxide. The third hypothesis was inconclusive as the assay failed. The readings of the ferrous sulfate samples were similar to those of the blank wells in the 96-well plates, meaning that no reaction occurred between the samples and the reagents. Finally, the fourth hypothesis was supported by the two behavioral assays in which fibromyalgia inflicted flies reacted similarly to the mimicking group when exposed to painful stimulus and the biochemical assays supported that the two groups would have different physiological makeups. This is important because of the lack of research into distinguishing what is and what is not fibromyalgia by current diagnostic tests such as the FM/a blood test. Nitric oxide's typical role as an inflammatory cytokine has made it an unsuspecting target

since fibromyalgia is a noninflammatory disorder, but in this study, NO is treated as a neurotransmitter for presynaptic membranes to become activated and initiate the cycle of pain stimulation. Overall, measuring nitric oxide's derivatives of nitrate and nitrite appear to be a promising path towards finding a physiological biomarker of fibromyalgia, however the relationship between arginase and nitric oxide needs further clarification.

Limitations

Limitations include the susceptibility of any project to human or machine error. Also, a lack of access to vertebrates and larger animals may have affected the ability to collect productive concentrations of nitric oxide in the ferrous sulfate samples. The project also has not been fully completed; thus a concrete conclusion cannot be made. Additions of other mutants that would model rheumatoid arthritis, have not been completed, however this addition would expand the applications of nitric oxide measurement upon diagnosis.

Error Analysis

In the nitrite/nitrate concentration assay, NOS, the positive control, was supposed to have the highest or at least significantly higher concentration than the negative control if it followed the hypothesis as intended. This is because the NOS group over-expresses the enzyme nitric oxide synthase, which, as shown in figure 1B, produces nitric oxide—the foundation of nitrite and nitrate. This may be due to a mistake made by the researcher while carrying out the assay, breeding the groups, and/or calculating the final values as well as machinery errors. As for the failure to achieve results in the "exhaled" nitric oxide concentration assay, there are a number of reasons this error may have occurred: the samples may not have had enough time to collect enough gas to be detectable, the nitrite/nitrate colorimetric assay may not have been suited for testing ferrous sulfate samples, or the hypothesis is not supported even if these supposed errors are addressed.

Future Research

To address the high false-positive rates of the FM/a blood test when testing on patients with RA or SLE, the addition of other mutants would help in the investigation of what physiologically makes each one of them different and how that information can be used for diagnosis. For the first stepping stone, rheumatoid arthritis can be modeled via the overexpression of the PAD4 gene. PAD4 produces proteins that convert arginine to citrulline, one of the main mechanisms of RA pathogenesis. This influx of citrulline causes anti-citrullinated protein antibodies to become activated and destroy tissue within joints, thus causing the pain often experienced in RA. Because of this, arginase is produced in increased volumes to combat the amount of arginine available for citrullination. This pathway has one specific function that makes it unique to FM: RA would increase arginase whereas FM would decrease it. Therefore, the difference in quantity would help separate the two upon diagnosis. Plus, by pinpointing large players in fibromyalgia and rheumatoid arthritis pathogenesis, targeting these areas for treatment will be most effective.

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