

The Effects of the Modulation by Melatonin on the Pathogenesis of Systemic Lupus
Erythematosus on *Drosophila melanogaster*

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ABSTRACT

There is a need for a cost-effective, safe, and accessible treatment for lupus. At least five million people around the world suffer from this disease. Systemic lupus erythematosus is a chronic autoimmune disease in which one's own tissue attacks itself. It can affect numerous areas of one's body and could even cause death. The effects of melatonin, a hormone that regulates the circadian rhythm, on immunity are known; however, its interactions with autoimmune disorders remains obscure.

In this experiment, three melatonin supplementations- direct melatonin, indirect melatonin as pistachios, and Serotonin N-acetyltransferase (NAT)- were fed to previously synchronized *Drosophila melanogaster* populations. The lupus indexes (IgG antinuclear antibodies, Interleukin 6, and cortisol) were used to assay for the effectiveness of the melatonin supplementations. The hypothesis was that the NAT variable will have the lowest lupus index levels. After conducting the experiment, the averages of the variables were analyzed, and it was found that the NAT variable had the lowest lupus index and highest melatonin levels, thus exemplifying that melatonin and the lupus indexes are inversely proportional. Furthermore, an ANOVA Statistics was tested along with Tukey's HSD follow-up, which supported the hypothesis. The direct melatonin was the second most effective variable, including the positive control; consequently, both of these supplementations could be considered viable treatments. Even though there are medications that can help treat lupus, they have limitations. These include the fact that melatonin is an economical alternative that is more attainable. Additionally, they have fewer side effects and do not cause addiction.

INTRODUCTION

A. Statement of Purpose

The purpose of this experiment is to determine how the different melatonin supplementations reduce the lupus indexes. The positive control will be ibuprofen and the negative control will be without any treatment, the dependent variable will be the melatonin indexes, and the independent variable will be the type of melatonin supplementation.

B. Background Research

At least five million people around the world suffer from lupus. The most common type, systemic lupus erythematosus (SLE), covers approximately seventy percent of all cases, and it is defined as a chronic autoimmune disease consisting of attacks on its own tissues (“Lupus facts and statistics”, 2016). Consequently, organs suffer from tissue damage and widespread inflammation. These organs include the brain, heart, lungs, kidneys, and skin. There is currently no cure for this disease, and 90% of all cases are women between the ages of fifteen and forty-five (Fava & Petri, 2012).

Systemic lupus erythematosus can have short-term and long-term effects on one’s life. It could limit a person’s physical, mental, and social functioning. Those who obtain an early diagnosis, however, along with effective treatments maybe be able to reduce SLE’s damaging effects and improve their quality of life; on the other hand, late diagnosis, lack of access to care or less effective treatments, and poor adherence to therapeutic regimens may increase these

effects which could result in an increase of complication and risk of death (Maidhof & Hilas, 2012).

Furthermore, SLE may be complicated to diagnose due to the fact that its early symptoms are not specific and could seem as if it were another disease. For instance, if a blood test was to be used singularly to diagnose this disease, there would be an increased risk of misdiagnosis. Thus, a combination of these tests would arise to the diagnosis. Blood and urine tests, such as the antinuclear antibody test, are more effective and can demonstrate if one's immune system is likely to make the lupus antibodies. With only this information, however, it is not enough to determine if one has lupus or not, so if these results are positive, the doctor would order more tests for antibodies more specific to SLE (Kuhn et al., 2015). Specifically, IgG, IgM, and IgA antinuclear antibody levels have been shown to be elevated in patients with SLE. Additionally, Interleukin 6 (IL-6) is an important cytokine related to inflammation and immune response, and cortisol is also interrelated with inflammation as well as one's sleep/wake cycle, both of whom's levels are shown to increase in patients with active SLE; thus, these are other prominent tests for lupus (Jost et al., 2014). Finally, skin or kidney biopsies could be performed. A standard biopsy can be defined as a minor surgery in order to remove a sample of living tissue which is then examined under a microscope. Specifically, the skin and kidney tissues can provide signs of an autoimmune disease (Cojocaru et al., 2011)

Melatonin is a hormone that is secreted from the pineal gland in the brain during the night. It has numerous functions including the regulation of the circadian and seasonal rhythms as well as antioxidant and anti-inflammatory effects. Even though it is known that this neuroendocrine system influences immunity, the interactions between hormones and autoimmune disorders remain obscure (Tordjman et al., 2017). Moreover, in the process of synthesizing melatonin, the enzyme Serotonin N-acetyltransferase (NAT) catalyzes the reaction from serotonin to melatonin. Pistachios have been found to have the highest levels of melatonin in regards to nuts. However, they can also be found in legumes, beverages, oils, yeast, and many more (Meng et al., 2017).

Drosophila melanogaster is a species of fruit flies commonly used for scientific research. This is due to the fact that they share 75% of the same DNA as humans, thus making them a very effective model for human biological studies as well as their analogy to more complex organisms and their highly conserved innate immune response. They are also easy to handle and reproduce rapidly. After the eggs are laid by the female fly hatch, it takes four days for the larvae, small white maggots that have a defined head and lack legs, to turn into a pupa, similar to a cocoon. During pupariation, a complete body metamorphosis from larva to adult occurs. In eclosion, adult flies emerge from the pupa case, which is tan-colored, three millimeters in length, and two millimeters wide. Finally, they develop into adult flies (Tolwinski, 2017).

The *hopscotch* gene is the *Drosophila* version of the Janus kinase (JAK) gene family in humans. This mutation is what is known as a gain of function mutation, meaning that the protein continues functioning even when it should be turned off. Thus, this causes the immune system to be “hyper-aware”, and similar mutations in people lead to autoimmunities such as lupus and leukemia (Zeidler & Bausek, 2013).

Therefore, in this experiment, different melatonin supplementations are administered to the mutant *Drosophila melanogaster* to simulate the treatment of lupus in humans.

C. Hypothesis

If the *Drosophila melanogaster* undergo different cycles, the ones with Serotonin N-acetyltransferase will have lower SLE levels.

METHOD

A. Materials

- Standard lab equipment
- Wild-type *Drosophila melanogaster*
- *Drosophila melanogaster* with SLE homolog (*hopscotch*)
- Melatonin
- Ibuprofen (Motrin®)
- *Drosophila* maintenance
- Homogenizer

- Dry ice and ethanol
- Centrifuge
- PBT (PBS 1X, 0.05 Triton X)
- Melatonin ELISA Assay Kit
- Cortisol ELISA
- IgG ANA ELISA
- IL-6 ELISA
- Microplate reader

B. Procedures

1. Breed *Drosophila*

Melatonin

1. Measure out the desired amount of distilled water using a graduated cylinder
2. Weigh out the desired amount of nutri-fly using a balance
3. Pour water into a beaker
4. Put the beaker on a hot plate and turn the heat to low
5. Gradually add the nutri-fly while stirring (or using a stir bar) and slowly incrementing the heat
6. Add 1mM melatonin to the nutri-agar
7. After the agar has boiled, take it off the hot plate
8. Once the temperature of the agar is below 60°C, pour around 20mL into each vial
9. Put the vials in the refrigerator until use

Serotonin N-acetyltransferase (NAT)

1. Measure out the desired amount of distilled water using a graduated cylinder
2. Weigh out the desired amount of nutri-fly using a balance
3. Pour water into a beaker
4. Put the beaker on a hot plate and turn the heat to low
5. Gradually add the nutri-fly while stirring (or using a stir bar) and slowly incrementing the heat
6. Add 1mM NAT to the nutri-agar
7. After the agar has boiled, take it off the hot plate
8. Once the temperature of the agar is below 60°C, pour around 20mL into each vial
9. Put the vials in the refrigerator until use

Indirect melatonin

1. Measure out the desired amount of distilled water using a graduated cylinder
2. Weigh out the desired amount of nutri-fly using a balance
3. Pour water into a beaker
4. Put the beaker on a hot plate and turn the heat to low
5. Gradually add the nutri-fly as well as the pistachio powder while stirring (or using a stir bar) and slowly incrementing the heat
6. After the agar has boiled, take it off the hot plate
7. Once the temperature of the agar is below 60°C, pour around 20mL into each vial
8. Put the vials in the refrigerator until use

Negative Control

1. Prepare nutri-agar
2. Measure out the desired amount of distilled water using a graduated cylinder
3. Weigh out the desired amount of nutri-fly using a balance
4. Pour water into a beaker
5. Put the beaker on a hot plate and turn the heat to low
6. Gradually add the nutri-fly while stirring (or using a stir bar) and slowly incrementing the heat
7. After the agar has boiled, take it off a hot plate
8. Once the temperature of the agar is below 60°C, pour around 20mL into each vial
9. Put the vials in the refrigerator until use
10. Keep flies in the food for 5 days

Positive Control

1. Measure out the desired amount of distilled water using a graduated cylinder
2. Weigh out the desired amount of nutri-fly using a balance
3. Pour water into a beaker
4. Put the beaker on a hot plate and turn the heat to low
5. Gradually add the nutri-fly while stirring (or using a stir bar) and slowly incrementing the heat
6. Add 1mM ibuprofen to the nutri-agar
7. After the agar has boiled, take it off the hot plate

8. Once the temperature of the agar is below 60°C, pour around 20mL into each vial
9. Put the vials in the refrigerator until use

Homogenization of the Drosophila

1. Flash freeze by pouring ethanol on dry ice.
2. Transfer tubes to -80 °C for storage until use.
3. Homogenize the Drosophila with PBT (PBS 1X, 0.05% Triton) and centrifuge (15 min at 4,000 x g, 4 °C).
4. Discard all disposable safety equipment in a biohazard container and sterilize all the other equipment.

Melatonin ELISA

1. Add 100 µL of the 1X Stabilizer to the B0 (0 ng/mL standard) wells and 150 µL to the NSB wells.
2. Add 100 µL of the standards and samples (10 trials) into the appropriate wells
3. Add 50 µL of the 1X melatonin tracer to all wells except for the blank.
4. Add 50 µL of the 1X melatonin antibody to all wells except for the NSB and blank.
5. Seal with plate sealer and incubate at room temperature.
6. Empty the contents of the wells and wash by adding full well volume, ~ 400 µL, of wash solution. Repeat the wash 2 more times for a total of 3 washes.
7. After the final wash, empty or aspirate the wells and firmly tap the plate on a paper towel to remove any remaining wash buffer.

8. Add 200 μ L of the melatonin conjugate solution to each well except the blank.
9. Seal the plate and incubate at room temperature
10. Add 200 μ L of TMB substrate solution into each well.
11. Seal the plate and incubate for 30 minutes. Add 50 μ L of the stop solution into each well.
12. Read the absorbance of the plate at 450 nm using a compatible microplate reader.

Cortisol ELISA

1. Add 150 μ L of Calibrator Diluent RD5-43 into the non-specific binding (NSB) wells.
2. Add 100 μ L of Calibrator Diluent RD5-43 to the zero standard (B0) wells.
3. Add 100 μ L of Standard and sample (10 trials) to the appropriate wells.
4. Add 50 μ L of Cortisol Conjugate to all wells. Wells will now be red in color.
5. Add 50 μ L of the Primary Antibody Solution to each well (excluding the NSB wells).

Cover with the adhesive strip provided. All wells will now be violet in color except the NSB wells, which will be red in color.
6. Incubate for 2 hours at room temperature, and aspirate each well and wash, repeating the process three times for a total of four washes.
7. Wash by filling each well with Wash Buffer (400 μ L) using a squirt bottle. Invert the plate and blot it against clean paper towels.
8. Add 200 μ L of Substrate Solution to each well. Incubate for 30 minutes at room temperature on the benchtop. Protect from light.
9. Add 50 μ L of Stop Solution to each well. The color in the wells should change from blue to yellow.

10. Read the absorbance of the plate at 450 nm using a compatible microplate reader.

IgG ELISA

1. Prepare 1:21 dilution of test samples, by adding 10 μ l of the sample to 200 μ l of sample diluent. Mix well.
2. Dispense 100 μ l of calibrator and controls into the appropriate wells. For the reagent blank, dispense 100 μ l sample diluent (10 trials). Tap the holder to remove air bubbles from the liquid and mix well.
3. Incubate for 20 minutes at room temperature. Wash wells three times with 300 μ l of 1X wash buffer. Blot on absorbance paper or paper towel.
4. Dispense 100 μ l of enzyme conjugate to each well and incubate for 20 minutes at room temperature.
5. Wash wells three times with 300 μ l of 1X wash buffer. Blot on absorbance paper or paper towel.
6. Dispense 100 μ l of TMB substrate and incubate for 10 minutes at room temperature.
7. Add 100 μ l of stop solution.
8. Read the absorbance of the plate at 450 nm using a compatible microplate reader.

IL-6 ELISA

1. Block wells with 200 μ L of ELISA/ELISPOT Diluent (1X). Incubate at room temperature for 1 hour.
2. Aspirate and wash at least once with Wash Buffer

3. Add 100 μ L/well of samples to the appropriate wells (10 trials), and 100 μ L of ELISA/ELISPOT Diluent (1X) to the blank well.
4. Seal the plate and incubate at room temperature for 2 hours or overnight at 4°C for maximum sensitivity
5. Aspirate and wash. Repeat for a total of 3-5 washes.
6. Add 100 μ L/well diluted Detection Antibody to all wells.
7. Seal the plate and incubate at room temperature for 1 hour. Aspirate and wash as in Step 2. Repeat for a total of 3-5 washes.
8. Add 100 μ L/well of diluted Streptavidin-HRP.
9. Seal the plate and incubate at room temperature for 30 minutes. Aspirate and wash. Repeat for a total of 5-7 washes.
10. Add 100 μ L/well of 1X TMB Solution.
11. Incubate at room temperature for 15 minutes, and add 100 μ L/well of Stop Solution.
12. Read the absorbance of the plate at 450 nm using a compatible microplate reader.

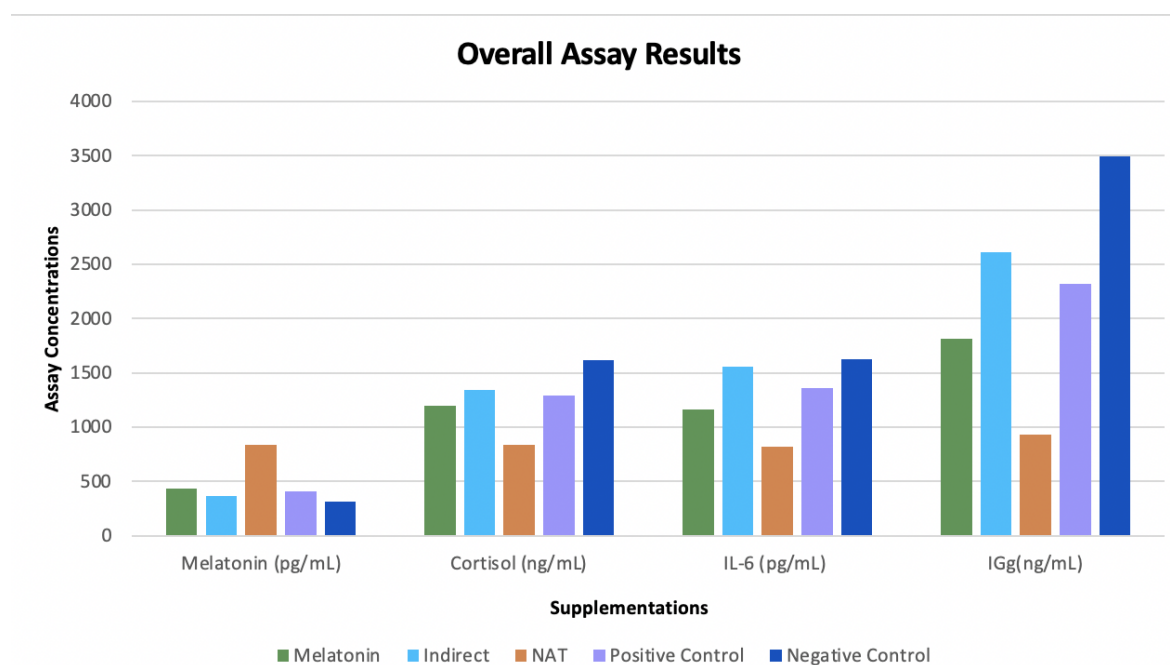
RESULTS

A. Chart

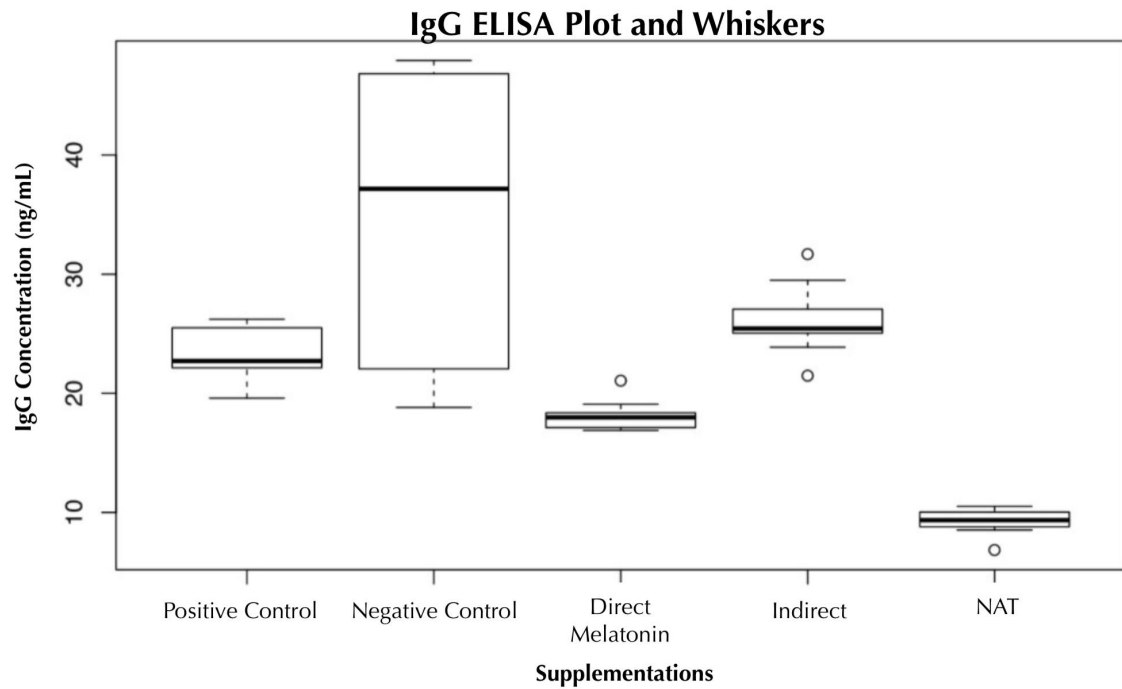
SUPPLEMENTATIONS						
ASSAYS	Melatonin	Indirect	NAT	Positive Control	Negative Control	Average
Melatonin (pg/mL)	430.8287716	368.8844143	838.72524	411.0852798	315.2852443	472.96179
Cortisol (ng/mL)	1199.380743	1346.730824	840.4099663	1287.773923	1620.260483	1258.911188
IL-6 (pg/mL)	1163.252508	1554.661371	817.5689799	1357.127926	1629.65092	1304.452341
IGg(ng/mL)	1813.690987	2613.133047	928.6266094	2322.918455	3493.991416	2234.472103
Above Average						
Below Average						

Chart of the overall data in the four assays. All NAT levels are below average except for the melatonin assay (explained in the Conclusion section).

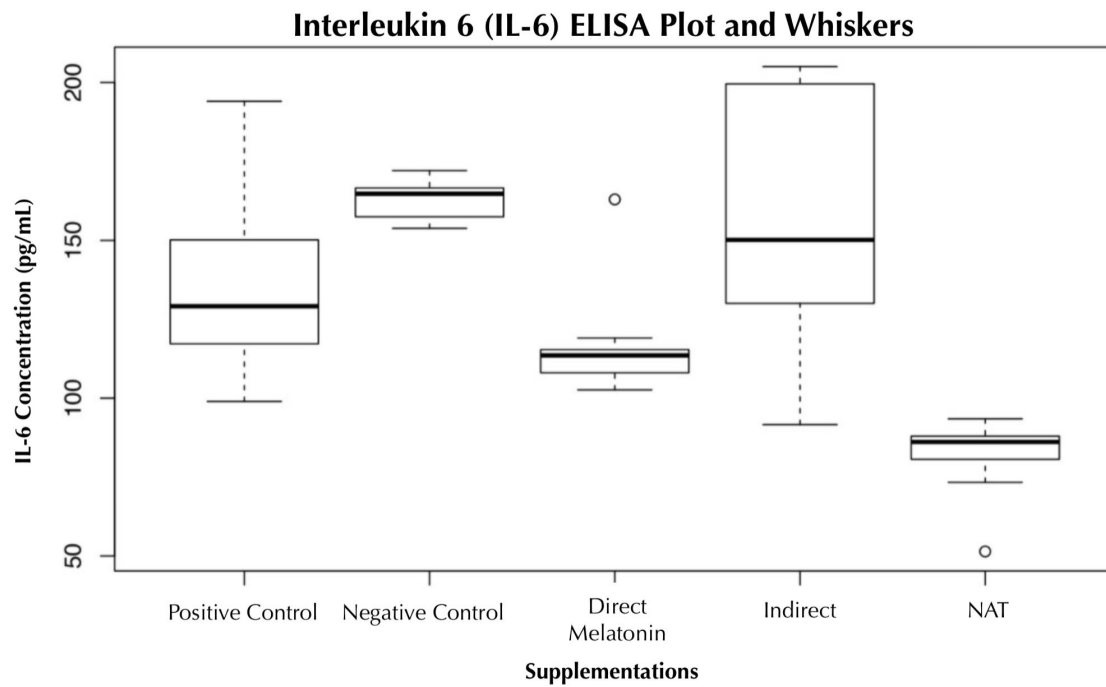
B. Graphs



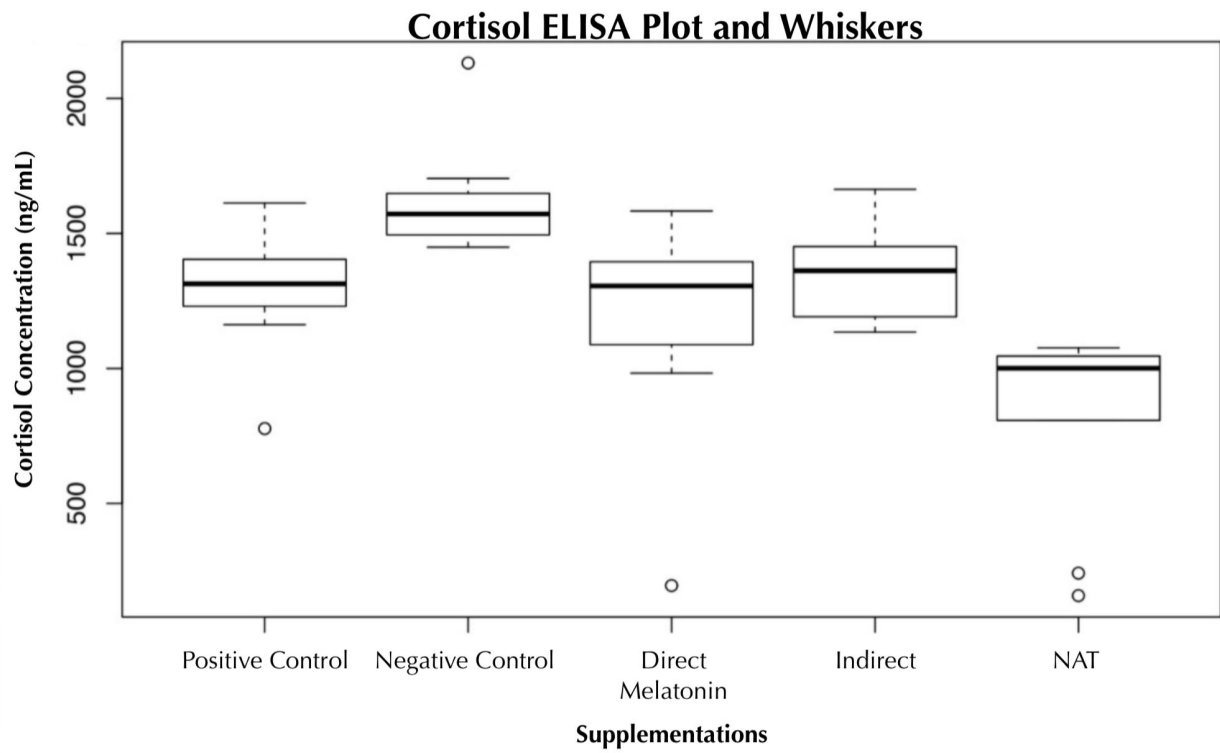
Bar Graph of overall data in the four assays.



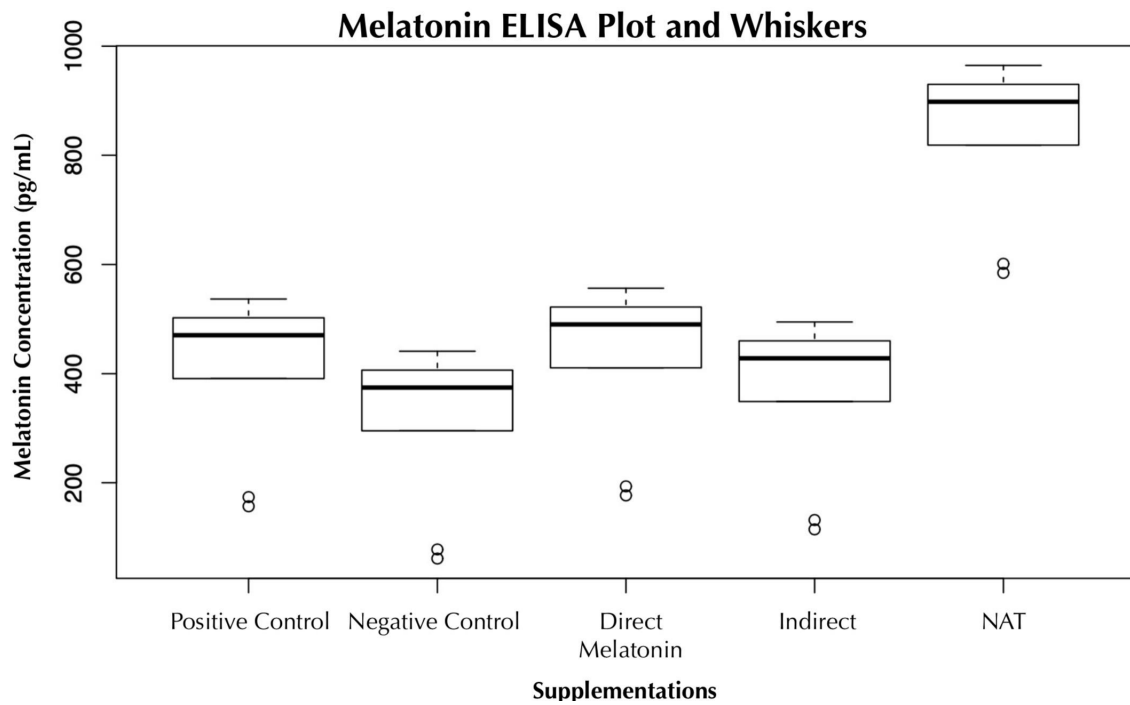
Plot and Whiskers graph of IgG displaying the distribution of data.



Plot and Whiskers graph of IL-6 displaying the distribution of data.

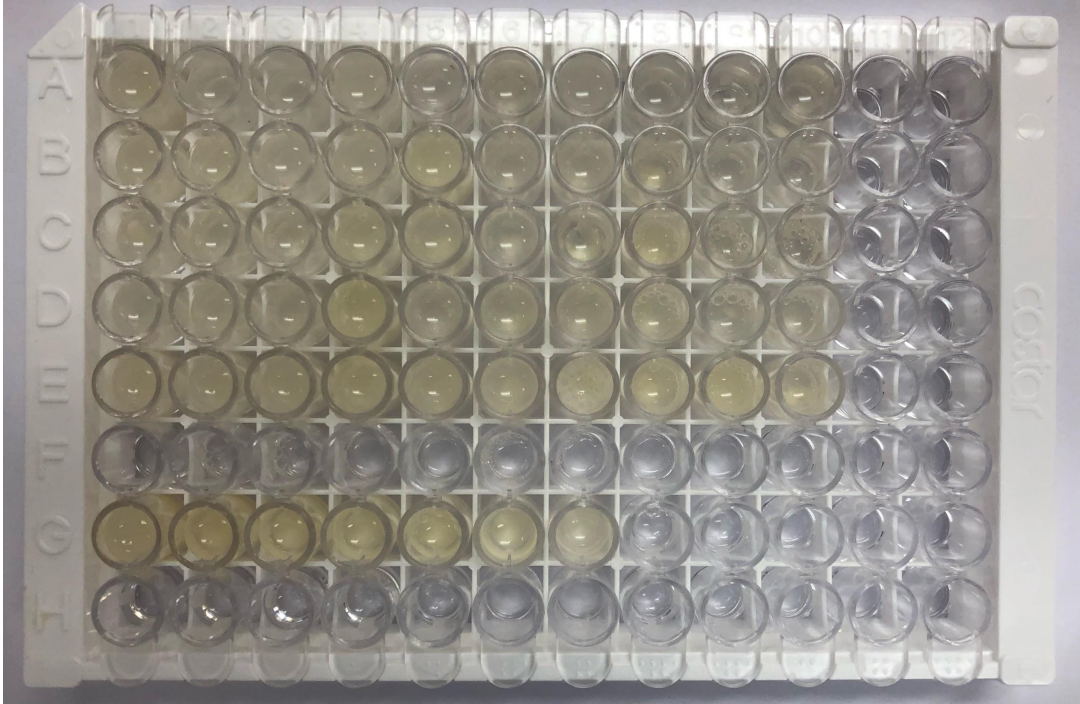


Plot and Whiskers graph of cortisol displaying the distribution of data.

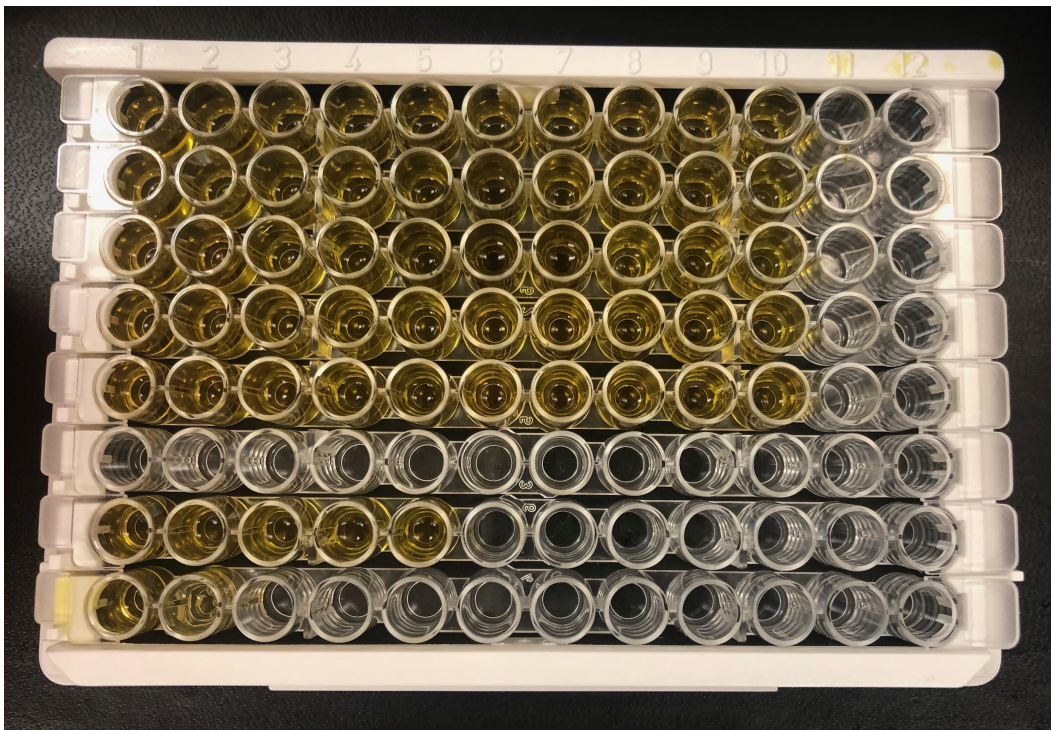


Plot and Whiskers graph of melatonin displaying the distribution of data.

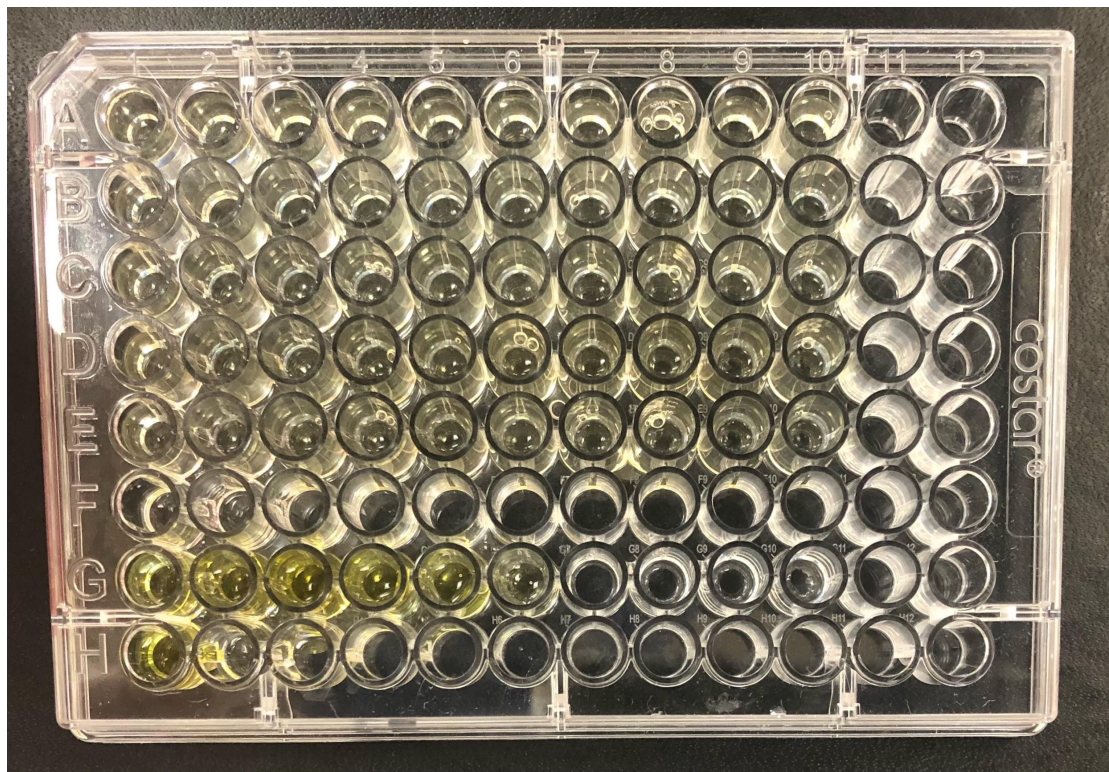
PHOTOGRAPHS



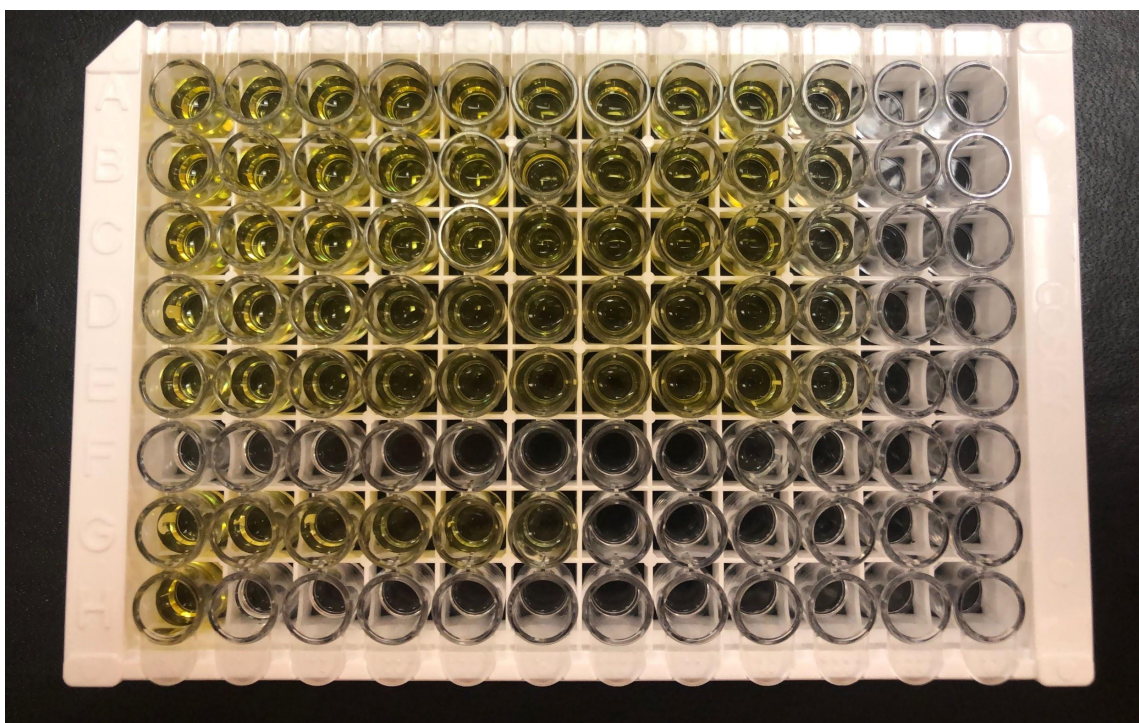
Cortisol ELISA 96-well plate. 1A-10A: Direct Melatonin, 1B-10B: Indirect, 1C-10C: NAT, 1D-10D: Negative Control, 1E-10E: Positive Control, 1G-7G: Standard Dilutions, 1H: Blank



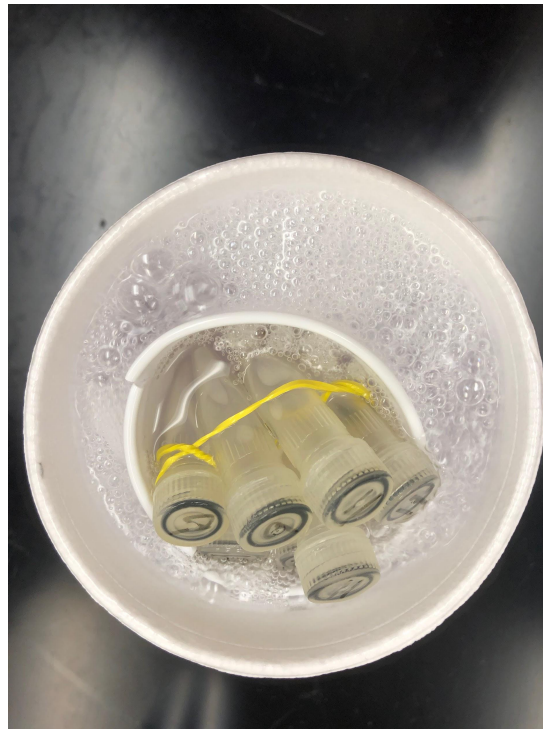
Melatonin ELISA 96-well plate. 1A-10A: Direct Melatonin, 1B-10B: Indirect, 1C-10C: NAT, 1D-10D: Negative Control, 1E-10E: Positive Control, 1G-5G: Standard Dilutions, 1H: Blank



IL-6 ELISA 96-well plate. 1A-10A: Direct Melatonin, 1B-10B: Indirect, 1C-10C: NAT, 1D-10D: Negative Control, 1E-10E: Positive Control, 1G-6G: Standard Dilutions, 1H: Blank



IgG ELISA 96-well plate. 1A-10A: Direct Melatonin, 1B-10B: Indirect, 1C-10C: NAT, 1D-10D: Negative Control, 1E-10E: Positive Control, 1G-6G: Standard Dilutions, 1H: Blank



Flash freezing the *Drosophila melanogaster*



Drosophila melanogaster under a microscope



Synchronizing the *Drosophila melanogaster*

D. Calculations

The raw data retrieved from the microplate reader was subtracted by the blank of each assay, and then a standard curve was traced whose values were multiplied against each dilution factor.

These results were analyzed by averaging the ten trials of each supplementation

(melatonin, indirect, and NAT) as well as the positive and negative control. They were placed in a chart (Figure 1) which included the four assays. Moreover, the values were extracted from the chart into a bar graph (Figure 2) where the five variables were compared within each assay.

Finally, a One-Way ANOVA with Tukey's Honest Significant Difference (HSD) follow-up, Figure 3, was conducted to find if there was any significant difference between NAT and the other variables.

DISCUSSION

A. Conclusion

The NAT supplementation demonstrated the most effective results when compared against the other variables- including the positive control- as shown in *Figure 2*. In order to support this, an ANOVA Statistics was tested along with Tukey's HSD follow-up. Due to the fact that the results were less than the P-value of 0.05, the hypothesis was supported.

In regards to the direct melatonin supplementation, it was found that it was the second most effective variable. When compared against the positive control, they were found to have relatively similar lupus index levels; nonetheless, the melatonin supplementation performed better in all of the assays. Even though there is a significant difference between the NAT and direct melatonin results, the latter could be considered a viable option. On the other hand, the indirect melatonin supplementation was the least effective treatment out of the three variables.

Moreover, the IgG levels appear to have been more affected overall compared to the other assays which concludes that there is a more direct relationship between IgG and lupus.

Due to the fact that NAT had the highest melatonin levels, contrasting the other assays, one can conclude that the melatonin levels are inversely proportional to the cortisol, IL-6, and IgG levels. The reason for this is that melatonin reduces the lupus indexes; thus, the more melatonin provided, the better performance.

B. Applications

Millions of people around the world suffer from lupus, and they can even die from it. Medicines to treat this disease do exist; however, there might be circumstances in which they are not able to take them. In underdeveloped regions, for instance, people die due to the lack of access to medicines; however, melatonin is a cost-efficient alternative that is easier to acquire which could help treat lupus in those locations. Even if the people were not able to find melatonin in their area, it is more likely that they would be able to purchase melatonin in a nearby place rather than the standard lupus medications. Furthermore, as opposed to other medications that are used for lupus, melatonin is not a prescription drug, thus facilitating the action of obtaining it. This supplement is cheaper than most other medications that a patient with lupus would have to take, thus making it more attainable to people, especially if they do not reside in those underdeveloped areas.

Moreover, people do not build up tolerance to melatonin, which usually tends to be a hallmark of addiction, and it does not cause people to exhibit symptoms of dependence. This

means that melatonin is not considered addicting, which is a trait that most lupus medications share. As a consequence, since there would not be a chance that they would become addicted to it, patients would be more likely to take melatonin without fear, which is sometimes the case for other medications. In addition, the other medications can have side effects. For instance, corticosteroids can cause acne, depression, increased risk of infections, osteoporosis, muscle weakness, among others. Even though there are also side effects to melatonin, these are less likely to be present.

C. Limitations

Despite the fact that the duration was based on literature previously analyzed, the duration that the *Drosophila* were in the supplementations could have affected the results. In a possible scenario, the melatonin could have continued to affect the flies and lowered the IgG, cortisol, and IL-6 levels further. Additionally, the number of trials- ten- as well as the amount of assays utilized, four, could have limited the usefulness of the results since lupus encompasses more than those parameters tested.

D. Error Analysis

When making the supplements, the meniscus of the graduated cylinder could have caused slight irregularities in the amount of water utilized in the foods. Another factor could be the fact that the scale utilized had two decimal places, thus there would not be an exact quantity being used. Also, since the Pistachio Diet contained a large amount of oil, the *Drosophila* repeatedly got stuck in it. Another reason for this could have been the temperature of the

environment. Consequently, a large amount of flies died, which caused the overall duration of the experiment to increase.

E. Future Research

Since the enzymes, NAT, were found to have the best results, possible future research could focus more on the precursors of the melatonin synthesis rather than just direct melatonin. Furthermore, based on the fact that the indirect supplementation (pistachios) has the lowest levels of melatonin among the variables, one could research whether it was due to the nut's own melatonin content or due to the body's metabolism and how it would be able to process this said indirect melatonin. Since human's melatonin levels and production are affected due to genetic and environmental conditions, which also affect their immune system response, this could lead to a more favorable environment for lupus, thus opening a field for further investigation. In this same way, flies could be affected by pre-existing genetic conditions and environmental aspects, such as light exposure and temperature. The next step would be to utilize a different model organism, such as mice, to confirm the finishing with the *Drosophila melanogaster*, and, eventually, humans where different demographic groups could be targeted as well.

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