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Article

Governing Glioblastoma: A novel therapy to restore motor control and mitigate glioblastoma proliferation

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SUMMARY

Glioblastoma multiforme (GBM) is a form of brain cancer with one of the poorest prognoses, including a 95% mortality rate over five years. We explored how dietary supplements can lower interleukin-6 (IL-6) levels and their associated seizure-like activity, a trademark of GBM progression. Specifically, we investigated how dietary supplements could regulate motor control in wild-type *Caenorhabditis elegans*. We hypothesized that if wild-type *C. elegans* are exposed to experimental heat-shock and treated with nitric oxide, *Andrographis paniculata* (green chiretta), or apigenin, that apigenin will most successfully restore motor control to mimic the wild-type strain. Apigenin directly interferes with the JAK-STAT and c-MET signaling transduction pathways most relevant to the pathogenesis of inflammation. We cultivated wild-type *C. elegans* in *Escherichia coli* before subjecting them to one dose of a nitric oxide booster and experimental heat shock. Subsequently, we treated the *C. elegans* with two dietary supplements that both reduce IL-6 levels: *A. paniculata* and apigenin supplements. After applying both the *A. paniculata* and apigenin supplements to their respective plates and monitoring the results in 24-hour intervals, we conducted the hard-touch test on all three plates to measure the thrash-to-touch ratio to classify the movement of the heat-shocked worms. Apigenin best mimicked the motor control of the healthy phenotype group with a high thrash-to-touch ratio of 3.511, whereas *A. paniculata* exhibited 2.8762 thrashes per touch and nitric oxide exhibited 2.3148 thrashes per touch. Apigenin's promising results reflect its potential as a more accessible therapeutic for GBM treatment.

INTRODUCTION

While other cancers have seen progress in developing treatments over the past few decades, glioblastoma (GBM), a spinal cord cancer, still has a high mortality rate. Traditional chemotherapy drugs are often unable to cross the blood-brain barrier (BBB) and thus largely remain ineffective in GBM (2). In GBM, mutations in astrocytes and oligodendrocytes cause cells to become malignant and invade healthy tissue (3). The highly invasive nature of GBM makes it extremely difficult for surgeons to remove without damaging healthy brain tissue (4).

Interleukin-6 (IL-6) is a proinflammatory cytokine that has multiple functions in the body (5). IL-6 plays a crucial role in regulating the body's immune response, including in GBM. In GBM and other cancers of severe grades, IL-6 is often overexpressed, leading to further tumor progression (6). IL-6 is critical for programmed death-ligand 1 (PD-L1) induction on myeloid immune cells, an important mechanism of immunosuppression (7). When IL-6 levels are lowered, both myeloid PD-L1 expression and the tumor growth rate are lowered (7). Additionally, IL-6 enhances signaling through the JAK-STAT3 pathway in GBM (6). Excess STAT3 activity stops healthy cell death, called apoptosis, and drives tumor cells to excessively multiply (6). Overall, IL-6 signaling promotes glioma growth by causing cells to become invasive and spread throughout brain tissue (6). Increased IL-6 levels are also strongly correlated with the increased seizure-like activity seen in GBM patients (8). Without access to observe intracellular effects of the supplements, it is most efficient to observe phenotypic changes through the presence of seizure-like activity. Thus, we decided to observe seizure-like activity presence in *Caenorhabditis elegans* as an indication of the effectiveness of the supplements.

Our research question centered around how dietary supplements can be used to regulate seizure-like activity in wild-type *C. elegans,* using heat-shock and nitric oxide as an inflammation stimulus (9). *C. elegans* are often used as a model organism for cancer research, including brain tumors like GBM due to its somatic genome that remains largely similar over time, allowing scientists to ascertain what alters normal cell proliferation (10). Dietary supplements are a more accessible alternative to chemotherapy because they are often cheaper and easier to obtain. Chemotherapy is expensive and energy-intensive, and dietary supplements can provide a potential respite.

Nitric oxide is a molecule that plays a critical role in exacerbating inflammation seen in abnormal phenotypes, indicated by excessive seizure-like activity, including in GBM (9). While nitric oxide plays a role in homeostasis, it can be damaging in high amounts (9). In general, under regular conditions, nitric oxide does not produce an inflammatory response, but in abnormal phenotypes it is overproduced and thus inflammatory (9). Altered neuronal and glial signaling can induce seizure-like activity, and nitric oxide has been known to exacerbate this process (11).

Apigenin, a drug that has a low toxicity, regulates key signaling molecules that trigger gliomagenesis (12). Apigenin lowered the production of a growth protein called TGF-β1 and inhibited excess activation of the c-Met pathway, which contributes to tumor growth (12). Apigenin is known to inhibit increased levels of IL-6; these elevated IL-6 levels have been

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associated with GBM progression (13). Additionally, apigenin is involved in the JAK/STAT signaling pathway, which regulates cellular response to inflammation (14). Apigenin is believed to decrease the levels of STAT3 phosphorylation (14). Research has shown that blocking the JAK2 and STAT pathway is effective in decreasing TNF-α–induced IL-6 production, which leads to reduced cancer growth (15). A study for airway epithelial cells utilized the NCI-H292 cell line, and showed that apigenin inhibited TNFα activity by blocking the NF-kB pathway (16). Because apigenin reduces inflated levels of IL-6, apigenin's vital role must be explored further.

A. paniculata (green chiretta) has been shown to lower IL-6 expression at the cellular level through regulating IL-6 signaling and STAT3 phosphorylation (17). *A. paniculata* also has been shown to induce healthy cell death, or apoptosis, without proving toxic to the cells (18). A GBM cell line, U251, when treated with *A. paniculata*, exhibited significantly lower tumor growth (18). *A. paniculata* also regulates cell apoptosis via c-Myc and p53 following cell cycle arrest in the G2/M phase (18). STAT3, Akt, and MAPK are pathways that *A. paniculata* affects positively (19). Because *A. paniculata* induces healthy apoptosis and has been shown to shrink tumor growth, its role in treating GBM must be investigated further.

The two dietary supplements that we used interfere with the signaling pathways that can cause the overexpression of IL-6. We hypothesized that if wild-type *C. elegans* are exposed to experimental heat-shock and treated with nitric oxide, and then with *A. paniculata* or apigenin, that apigenin would most successfully restore motor control to mimic the wild-type strain. This is because apigenin directly interferes with the signaling transduction pathways most relevant to the pathogenesis of inflammation (1). When the *C. elegans* are thrashing, they cannot control their motor movements, also often seen in seizure-like activity. They have deteriorating motor control, which we wanted to investigate.

The apigenin supplement more effectively restored the motor control of the *C. elegans* compared to the *A. paniculata* and nitric oxide supplements, as the thrash-to-touch ratio was higher for the group exposed to the apigenin supplement post heat-shock. Thus, apigenin has potential as a supplement in GBM treatment.

RESULTS

We observed *C. elegans* for seizure-like activity after treatment with nitrous oxide, *A. paniculata*, or apigenin. Seizure-like activity was observed by looking for a low thrash-to-touch ratio, signifying their loss of ability to move freely – often seen in seizure-like activity. *C.* elegans were exposed to nitric oxide and then *to A. paniculata* or apigenin depending on the trial. We stroked the tail ganglia of the *C. elegans* with an eyelash, as the tail ganglia has one of the largest clumps of nerve cells, to determine the thrash-to-touch ratio (20). We measured the thrash-to-touch ratio by counting how many times the *C. elegans* thrashed per stroke. We conducted five trials per condition, one trial each week.

Negative control

We utilized wild-type *C. elegans* not exposed to any experimental heat-shock as a baseline for a healthy phenotype. The *C. elegans* exhibited a mean thrash-to-touch ratio of 3.99, 3.594, 2.921, 3.36, and 4.029 across the five subsequent trials **(Figures 1-3).** Throughout the five trials, the thrash-to-touch ratio of the *C. elegans* remained above the benchmark for a healthy phenotype, 3 thrashes per touch, excluding the thrash-to-touch ratio of 2.921. This anomaly was likely due to human error.

Positive control

The *C. elegans* in the positive control group were exposed to heat-shock, but not to the experimental nitric oxide booster. These *C. elegans* experienced seizure-like activity, although not as many as the group exposed to only the nitric oxide booster. Throughout the five trials, the *C. elegans* in the positive control group exhibited a mean of 2.408 thrashes per touch. **(Figures 1-3)**. The positive control group was statistically significant compared to the negative control group. The mean thrash-to-touch ratio was significantly different between the positive and negative control groups. The means were 2.408 and 3.99 thrashes per touch respectively ($p = 0.05$, one-way ANOVA).

Nitric oxide: Secondary positive control

The *C. elegans* exposed to the nitric oxide booster was a secondary positive control and exhibited exacerbated seizure-like activity after previous experimental heat-shock. The group exposed to the nitric oxide booster exhibited a mean of 2.3148 thrashes per touch averaged over the five subsequent trials **(Figure 1)**. The mean thrash-to-touch ratio was statistically significant between the nitric oxide groups, 2.3148, and the negative control groups, 3.99 ($p = 0.05$, one-way ANOVA).

Andrographis paniculata: Lesser effective supplement

The *C. elegans* exposed to the *A. paniculata* supplement facilitated the *C. elegans* to regain some of their motor control after exposure to previous experimental heat-shock with a combination of both treatments. The *C. elegans* exhibited a mean thrash-to-touch ratio of 2.9482 across the

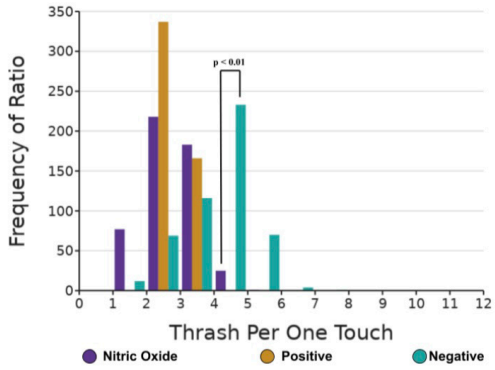

Figure 1: Nitric oxide thrash-to-touch ratios. Thrashes induced by the hard-touch test in *C. elegans* across the five trials after being treated with the nitric oxide as well as the negative and positive controls (N=1,506). *C. elegans* were treated with 50 uL of nitric oxide, then heat shocked to exacerbate seizure-like activity. The ranges in the graph depict the various thrash-to-touch ratios that the *C. elegans* exhibited, from 0 thrashes-per-touch to 12 thrashes-per-touch. Throughout the five trials, the highest mean observed was nitric oxide with 3.586 thrashes per touch, followed by the positive control with 2.41 thrashes per touch. The least thrashes observed were with the nitric oxide with 2.31 thrashes per touch. The nitric oxide had a p value of 0.01 compared to the positive control group.

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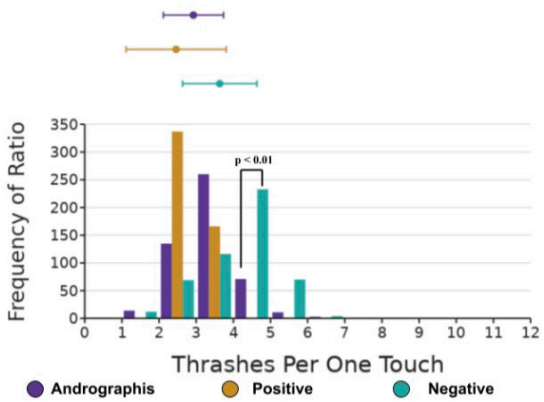

Figure 2: *A. paniculata* thrash to touch ratios. Thrashes induced by the hard-touch test in *C. elegans* across the five trials after being treated with the *A. paniculata* as well as the negative and positive controls (N=1,506). *C. elegans* were treated with 50 µL of nitric oxide, then heat shocked to exacerbate seizures, and after a 24-hour interval, 50 uL of *A. paniculata* was put on each plate. The ranges in the graph depict the various thrash-to-touch ratios that the *C. elegans* exhibited, from 0 thrashes-per-touch to 12 thrashes-per-touch. Throughout the five trials, the highest mean observed was nitric oxide with 3.586 thrashes per touch, followed by *A. paniculata* with 2.877 thrashes per touch. The least thrashes observed were with the positive control with 2.41 thrashes per move. The *A. paniculata* matched pair groups had a p value of 0.01 compared to the positive control group.

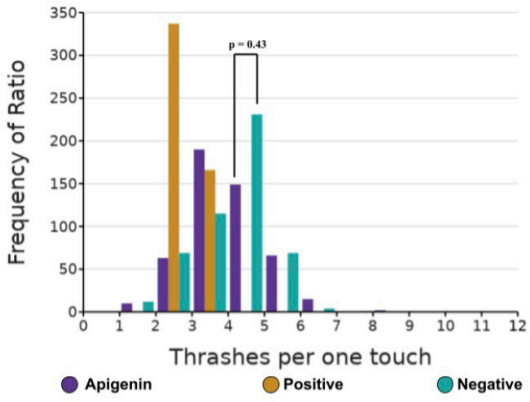

Figure 3: Apigenin thrash to touch ratios. Thrashes induced by the hard-touch test in *C. elegans* across the five trials after being treated with the apigenin as well as the negative and positive controls (N=1,506). *C. elegans* were treated with 50 uL of nitric oxide, then heat shocked to exacerbate seizures, and after a 24-hour interval, 50 uL of apigenin was put on each plate. The ranges in the graph depict the various thrash-to-touch ratios that the *C. elegans* exhibited, from 0 thrashes-per-touch to 12 thrashes-per-touch. Throughout the five trials, the highest mean observed was negative control with 3.586 thrashes per touch, followed by apigenin with 3.511 thrashes per touch. The least thrashes observed were with the positive control with 2.41 thrashes per move. The apigenin matched pair had an p value at 0.43 compared to the positive control group.

five subsequent trials **(Figure 2).** The mean thrash-to-touch ratio was statistically significant between the *A. paniculata* groups. The means were 2.9482 and 3.99 thrashes per touch respectively (p = 0.05, one-way ANOVA).

Apigenin: Most effective supplement at restoring thrash-to-touch ratio

The *C. elegans* exposed to the apigenin supplement retained motor control not statistically significant from the negative control group. The *C. elegans* exhibited a mean thrash-to-touch ratio of 3.511 averaged across the five subsequent trials **(Figure 3)**. The mean thrash-to-touch ratio varied between 2.99-3.848 thrashes per touch. The apigenin matched pair had an p-value at 0.43. The administration of apigenin helped facilitate recovery of motor control to most closely mimic the negative control with a healthy phenotype.

DISCUSSION

Overall, we wanted to investigate how seizure-like activity could be mitigated by herbal supplements. We heat-shocked *C. elegans* to exhibit seizure-like activity and exacerbated this activity with nitric oxide. We then tested apigenin and *A. paniculata* to see which group had a thrash-to-touch ratio that matched the negative control group. We found that the group treated with apigenin had a thrash-to-touch ratio that was most similar to the negative control group, 3.511 compared to 3.99. The motor control of the *C. elegans* treated with the apigenin supplements is similar to the motor control of the *C. elegans* in the negative control group. The experimental group that was exposed to the apigenin supplement regularly exhibited a mean thrash-to-touch ratio of above 3:1, depicting the lack of seizure-like activity, which would render the thrash-to-touch ratio between 2 - 2.9. The experimental group exposed to the apigenin supplement was not statistically significant from the negative control group. Both the groups that were exposed to the *A. paniculata* supplement and nitric oxide booster showed statistically significant from the negative control group, although the nitric oxide booster group had a lower thrash-to-touch ratio than the *A. paniculata* group. The group treated with *A. paniculata* had a mean thrash-to-touch ratio of 2.9482, which is lower than the apigenin's group of 3.511 thrashes per touch. Both apigenin and *A. paniculata* improved the thrash-to-touch ratio, but apigenin was a more effective herbal supplement than *A. paniculata.*

A low thrash-to-touch ratio indicates a loss of motor controls and increased seizure-like activity. The experimental group exposed to nitric oxide maintained a thrash-to-touch ratio above two thrashes per touch, never exceeding three thrashes per touch. When compared back to the experimental group that received the heat-shock treatment but no supplement, it can be ascertained that the nitric oxide booster exacerbated the seizure-like activity and induced a loss of motor control. Exposure to the nitric oxide supplement influenced *C. elegans* to become stagnant, with some unable to register any external stimuli. The *C. elegans* treated with nitric oxide had a reduced thrash-to-touch ratio than the negative control group, signifying nitric oxide causes an increase in *C. elegans'* seizure-like activity. The mean thrash-to-touch ratio for the positive-control group was lower than the group exposed to the nitric oxide booster post heat-shock, indicating how the nitric oxide exacerbated the C. elegans' seizure-like activity. We found a higher thrash-to-touch ratio for the positive control compared to the nitric oxide booster group, but a lower thrash-to-touch ratio when compared to the negative control group, the apigenin group, and the *A. paniculata* groups.

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The c-MET/HGF pathway has been shown to exacerbate tumor growth by blocking apoptosis when overexpressed (24). A previous study has shown that high c-MET expression is present in patients with GBM (24). Researchers observed that apigenin blocked the activation, or phosphorylation of the c-MET pathway which led to a decrease in cell proliferation due to inactivation of certain inflammatory proteins that were activated through the pathway (14). Moreover, apigenin has shown to induce apoptosis by activating the MAPK/ERK pathway which plays a role in cell differentiation and cell death depending on the cell stimulus. Apigenin lowers anti-apoptotic proteins present which encourage rampant cell proliferation, like Bcl-xL and Bcl-2, while increasing the amount of apoptotic proteins like BAX to encourage healthy cell death that prevents cancer (14). In general, apigenin works to block pathways that interfere with apoptosis, while also promoting the ones that support apoptosis.

The G2/M phase is key in cell growth, and *A. paniculata* works by halting the cell cycle at this phase, preventing damaged cells from dividing further (25). Moreover, *A. paniculata* targets the ERK1 signal-regulated kinase which is involved in inducing apoptosis (18). However, unlike apigenin, *A. paniculata* does not work directly with the pathways that are involved with GBM specifically. Apigenin works directly with the MAPK and NF-Kb pathway, which are two of origin sites for glioblastoma proliferation (25, 26).

While both the drugs used in this study show promising results as incorporation into modern pharmacology usage, apigenin proves consistency. Moreover, from their mechanisms, apigenin directly targets the main issue of reducing IL-6 levels while *A. paniculata* works mainly to reinstate apoptosis. Thus, apigenin may prevent downstream effects of IL-6 like seizure-like activity. *A. paniculata* may provide fewer solutions while apigenin works to prevent IL-6 downstream effects by interfering with the upstream activation. *A. paniculata* (green chiretta), on the other hand, works with DNA damage and suspends the cell from passing the G2 checkpoint (25). While this is effective in preventing the replication of damaged DNA, the signaling pathway is a more direct approach considering the specialization of GBM.

Due to contamination from black mold, the petri dishes had to be remade mid-trials, but those trials were disregarded and new ones were immediately made. The trials were redone with the new plates and the old data was discarded. When E. coli growth was minimal, the plates were also remade and the trial was restarted. All the procedures and materials remained the same otherwise. The information in the above sections follows the successful repeat of the experiment and not its initial attempt.

In the future, we want to directly manipulate the IL-6 levels to monitor its effects on seizure-like activity. Directly manipulating the IL-6 levels will allow us to observe specific effects of manipulated IL-6 levels on seizure-like activity and GBM cell proliferation. We will be able to study this through gene editing, and cell lines.

Every year, people continue to be diagnosed with GBM, and a treatment is yet to be found. Combating this disease and pursuing more research can serve as a blueprint for further research and is crucial for thousands of families. This research serves to contribute to the ongoing search for new developments by providing an option of incorporating apigenin as an ingredient for pharmaceutical studies. While it's unlikely to treat cancer with only simple herbs, they could be used alongside pharmaceutical advancements in the right concentrations, in order to boost the gentler properties of the treatment. Taking these results into consideration, more research into holistic medicine proves to be a promising strategy in the fight against GBM.

MATERIALS AND METHODS

Nematode growth and collection

A 2% w/v solution of Nematode-Growth Medium (NGM) Lite Agar (Bio-Rad Cat#1665125) was prepared. This solution was boiled until a homogenous solution was formed and then poured into petri dishes until they were halfway full and allowed to cool for 15 minutes until a jelly-like consistency was achieved. Once the plates had cooled, 150 µL of OP50 *E. coli* (BioZ Cat # 23723) were spread with an inoculating loop across the plates. Plates were then placed in an incubator at 37°C for 24 hours to allow the *E. coli* to grow.

A M9 wash buffer was created by mixing 3 g of KH_2PO_4, 6 g of Na_2HPO_4, and 5 g of NaCl in 1 L of H_2O. The mixture was then autoclaved for 20 min before 1 mL of 1 m $MgSO_4$ was added. The final buffer was stored at room temperature and checked for visible contamination before use. To collect *C. elegans* for plating, 1 mL of the M9 buffer was pipetted onto the stock pate containing N2-Wildtype *C. elegans* (Carolina™ Cat # S07601ND). From the stock plate, 200 µL of the solution was then pipetted onto the 5 experimental plates (apigenin, *A. paniculata)*, nitric oxide, positive control, negative control). The plates were stored at room temperature (21°C) for 24 hours.

Preparing experimental solutions

One capsule (2250 mg) of a nitric oxide booster supplement containing L-Arginine, L-Arginine HCl, Arginine AKG 2:1, L-Citrulline, L-Citrulline Malate 2:1 (Nutricost Cat # B09B4DWNYQ) was dissolved into 225 mg of distilled water to create a 10:1 solution. One capsule (400 mg) of an *A. paniculata* supplement (Nature's Way Cat # B0002I6Q30) containing 300 mg *A. paniculata* extract lead and stem standardized to 10% andrographolides 30 mg, and 100 mg *A. paniculata* (stem, leaf flower) was dissolved in 40 mg of distilled water to create a 10:1 ratio. One capsule (300 mg) of an apigenin supplement containing 100 mg apigenin and 200 mg L-Theanine was dissolved in 30 mg of distilled water to create a 10:1 ratio.

Application of nitric oxide booster and heat-shock

After the N2 wildtype *C. elegans* have sat for 24 hours, 50 µL of the 10:1 nitric oxide to water solution was pipetted onto the experimental plates, excluding both the positive and negative controls. The three plates were then exposed to experimental heat-shock in an incubator at 37ºC for 1 hour. The three *C. elegans* plates were then placed under a microscope and recorded in 2-minute intervals.

Application and measurement of *A. paniculata* and apigenin supplements

A sterile eyelash was obtained and attached to a toothpick via tape in order to measure the *C. elegans'* thrash-to-touch ratio when exposed to external stimuli, and it was then compared to the benchmark which was calculated prior to the study. In the three plates exposed to the nitric oxide and the

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experimental heat-shock, the tail ganglia of the *C. elegans* was stroked and the forward thrashes (a thrash to the left and a thrash to the right equates one complete thrash) were recorded over a 2-minute interval. The number of strokes was measured along with the number of thrashes. The data was inserted into a chart, where the number of thrashes were compared to 1 stroke, or exposure to the external stimuli. Next, 50 µL of both the 10:1 *A. paniculata* and apigenin to water supplement solutions were pipetted onto 2 of the plates that had undergone the experimental heat-shock and exposure to the nitric oxide booster to ascertain motor control recovery and seizure-like activity decline. The plates sat at room temperature (21°C) for 24 hours before the next measurements were taken. The worms were recorded over a 2-minute interval. Subsequently, the thrash-to-touch ratio for both the plates across a 2-minute interval was counted and inserted into a chart. This procedure was repeated across all 5 trials for the most accuracy and consistency. We ran multiple statistical tests to validate the results. To analyze statistical differences between our experimental groups, the one-way ANOVA statistical test was used to ascertain the signal against the noise to determine if the data was statistically significant. The following denotes our null and alternative hypotheses for each of the treatment groups: apigenin, *A. paniculata*, and nitric oxide. We used the F-statistic to conduct the Post-hoc Tukey's Range test as a matched pair T-test to understand the differences between the treatment and control groups.

Statistics

To analyze statistical differences between our experimental groups, we used a one-way ANOVA. The F-statistic (MS/MS Residual) for our data is 73. The significance level we tested against was $\alpha = 0.05$.

We also conducted a post-hoc Tukey's Range Test to determine the mean difference between the treatment groups and the control groups. This was conducted as a matched pair T-test for multiple groups. Further, the *p*-value of each group was assessed against a significance value of 0.05.

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