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Virulence Factor of *Porphyromonas gingivalis* Disrupts Intestinal Oxidative Status and Acetylcholinesterase Activity in Rotenone-Exposed Zebrafish

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ABSTRACT

Objective: *Porphyromonas gingivalis* (*P. gingivalis*) is a major pathogenic bacterium in periodontal disease and is associated with neurodegenerative diseases. One of the most destructive endotoxins of *P. gingivalis* is gingipain. Our objective was to show the impact of gingipain on acetylcholinesterase (AChE) activity and oxidative status in the intestinal tissues of zebrafish exposed to rotenone.

Materials and Methods: Zebrafish were grouped as; control group (C), gingipain-injected group (G), rotenone-exposed group (R), gingipain-injected and rotenone-exposed group (G+R) (n=15). At the end of 4 weeks, spectrophotometric analyses were performed to evaluate the oxidative status and AChE activity in the intestinal tissues.

Results: Intestinal lipid peroxidation (LPO) levels were higher in the G group than in the C group. Gingipain injection significantly reduced the activities of AChE and superoxide dismutase (SOD). In the R group, there were significant elevations in SOD, nitric oxide (NO), glutathione-S-transferase (GST), and AChE activities compared with those in the C group. In the G+R group, LPO, NO, SOD, and GST activities were reduced compared with the R group.

Conclusion: Our results show that gingipain dysregulated AChE activity and the oxidant-antioxidant balance in rotenone-exposed zebrafish, demonstrating its possible role in gut dysbiosis, neuroinflammation, and Parkinson's disease.

Keywords: *Porphyromonas gingivalis*, gingipain, zebrafish, rotenone, oxidative stress, acetylcholinesterase

INTRODUCTION

Approximately 11.2% of the world's population is affected by periodontitis, which is a disease characterized by the destruction of the tissues that surround the teeth (1). *Porphyromonas gingivalis (P. gingivalis)* is a key pathogen for periodontitis progression and has many virulence factors, including fimbriae, lipopolysaccharide (LPS), capsule, and cysteine proteases to overcome the host defense.

Gingipain, cysteine proteinases from the trypsin-like enzyme family, are destructive virulence factors of *P. gingivalis*. They are categorized into two main groups: lysine-specific gingipain (Kgp) and arginine-specific

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gingipain (RgpA, RgpB) (2). They execute a significant portion of the proteolytic activity that *P. gingivalis* utilizes in periodontal tissues to overcome the host defense. They negatively affect the functioning of extracellular matrix components, disrupt the structure of cell metalloproteinases, and dysregulate the host defense. The release of various compounds into the extracellular matrix is facilitated by gingipain by breaking down collagen and proteins in the cell structure, contributing to the nutrition and proliferation of bacteria. They reduce the effects of immune cells by causing impairment in T cell receptors, such in $CD4^+$ and $CD8^+$ T cells, to overcome the host's immune response (2).

The detrimental consequences of *P. gingivalis* and gingipain are not limited to periodontal tissues. *P. gingivalis* and its endotoxins can disseminate to various organs through circulation and the digestive tract, thereby contributing to the progression and severity of many diseases, including cardiovascular diseases, diabetes mellitus, rheumatoid arthritis, and neurodegenerative diseases (3, 4). For instance, *P. gingivalis* was detected in a study examining post-mortem brain tissue samples from individuals with Alzheimer's disease (3). Moreover, gingipain was identified in the plasma samples of Parkinson's disease (PD) patients (5).

Among neurodegenerative diseases, PD ranks as one of the most prevalent globally. Its characteristic feature is the deterioration of dopamine-producing neurons, subsequently affecting many dopamine-required motor and non-motor functions in the body. Dysfunctions of the gastrointestinal system are prevalent non-motor symptoms of PD, and some recent research suggests that PD progression has been linked with changes in intestinal homeostasis through a structure of neurons named the enteric nervous system (ENS) (6).

The sophisticated neuronal network located along the lining of the gastrointestinal tract (GIT) is known as the ENS, also frequently called "the second brain". It has essential functions for maintaining homeostasis in GIT by regulating various processes, including secretion, peristalsis, and absorption (7). During these processes, a diverse intestinal microbial community produces chemicals that modulate ENS activity and contribute to proper digestion. There are constant molecular interactions between the ENS and the central nervous system (CNS) through hormones, vagus nerve, immune cells and neurotransmitters like acetylcholine and dopamine. This interconnection between these two systems is frequently termed the "gut-brain axis" (6).

Acetylcholine (ACh) is a neurotransmitter and a neuromodulator that regulates dopamine, serotonin, and other neuro hormones for a balanced cholinergic system in the CNS. In addition, ACh has an anti-inflammatory function via the "cholinergic anti-inflammatory pathway" which involves the inhibition of pro-inflammatory cytokines. ACh plays an essential role in ENS functioning by signaling digestive enzyme secretion, promoting peristalsis, and smooth muscle contraction. Acetylcholinesterase (AChE) is an enzyme that regulates ACh levels by modulating its activity and contributes to the maintenance of proper intestinal function (8). Imbalances in ACh/AChE activities have been linked with irritable bowel syndrome (IBS) and inflammatory bowel disease, highlighting their importance in maintaining intestinal homeostasis (9).

Disturbances in intestinal homeostasis, such as dysbiosis in microbial composition, can lead to increased reactive oxygen species (ROS) levels and cause oxidative stress in the GIT. The changes within the oxidant-antioxidant molecules alter the oxidative balance and affect intestinal homeostasis. Recently, there has been an increasing amount of attention in studies that focus on the possible connection between disturbances in the gut microenvironment and neurodegenerative diseases via the gut-brain axis. Accordingly, recent research has indicated that pathological changes associated with PD can also be present in the enteric nervous system, contributing to disease progression (6).

The research focused on revealing the underlying mechanisms of PD is continuing with human and animal studies. Zebrafish is an exotic freshwater fish that is a preferred model for PD studies because of its similarities to the human immune system and the well-characterized dopaminergic system. Rotenone, a member of the rotenoid family of chemicals, is one of the most commonly used neurotoxins for inducing PD in animals (10). It accumulates in neurons and inhibits mitochondrial complex I, which elevates ROS levels and leads to neuron dysfunction. When zebrafish are exposed to rotenone, their dopaminergic neurons are damaged, resulting in reduced dopamine levels, impaired motor function, behavioral abnormalities, and intestinal dysfunction associated with PD (10). In recent years, it has been proposed that *P. gingivalis* along with its virulence factors, especially gingipain, may change the gut microbiota and possibly be related to neurodegeneration via the gut-brain axis. To elucidate the impact of gingipain on intestinal homeostasis and to understand its link to neurodegenerative diseases, we directly administered gingipain during rotenone exposure in a zebrafish model. Our study evaluated gingipain's effect on the gut AChE activity and oxidant-antioxidant status in rotenone-exposed zebrafish.

MATERIALS AND METHODS

Animal Experiments

The experiments conducted in this study followed the guidelines outlined by the European Communities Council Directive of November 24, 1986 (86/609/EEC). The study's techniques were approved by the Animal Care and Use Committee of Marmara University (17.2022mar). The standards of Animal Research: Reporting of *in vivo* experiments were followed, and every attempt was made to use the fewest number of animals in the study as possible.

AB/AB strain, male/female, wild type, 4-6 months old, healthy zebrafish (*Danio rerio*) were maintained in an aquarium setup (ZebTEC, Italy) that was adjusted to 27–28 ± 1 °C under a

14/10 h light/dark period. The fish were given flake fish food (20 mg) twice daily (Tetramine, Germany). Sixty adult zebrafish were divided into four groups randomly. The groups were assigned as follows: Control (C); Gingipain (G); Rotenone (R); Gingipain + Rotenone, (G+R) (n=15 each) groups. To replicate the systemic inflammatory effect of periodontitis, zebrafish in the G group received 93 nmol/L gingipain (MyBioSource, United States, Recombinant *P. gingivalis* Gingipain, RgpA, MBS969681) injections intraperitoneally. Gingipain concentration was established by our research group's previous study (11). The injections were administered by the same researcher and performed once every 7 days for 4 weeks. In 5 L of aquarium water, the R group was exposed to a mixture of 5 µg/L rotenone (Sigma, United States) and dimethyl sulfoxide (0.1%) (Sigma, United States), based on our group's prior studies (12). Fish in the G+R group underwent intraperitoneal injections of gingipain (93 nmol/L), and were exposed to rotenone (5ug/L). The zebrafish in the control group were injected intraperitoneally with 5 µL phosphate-buffered saline to mimic the stress caused by the injection procedure in the experimental groups.

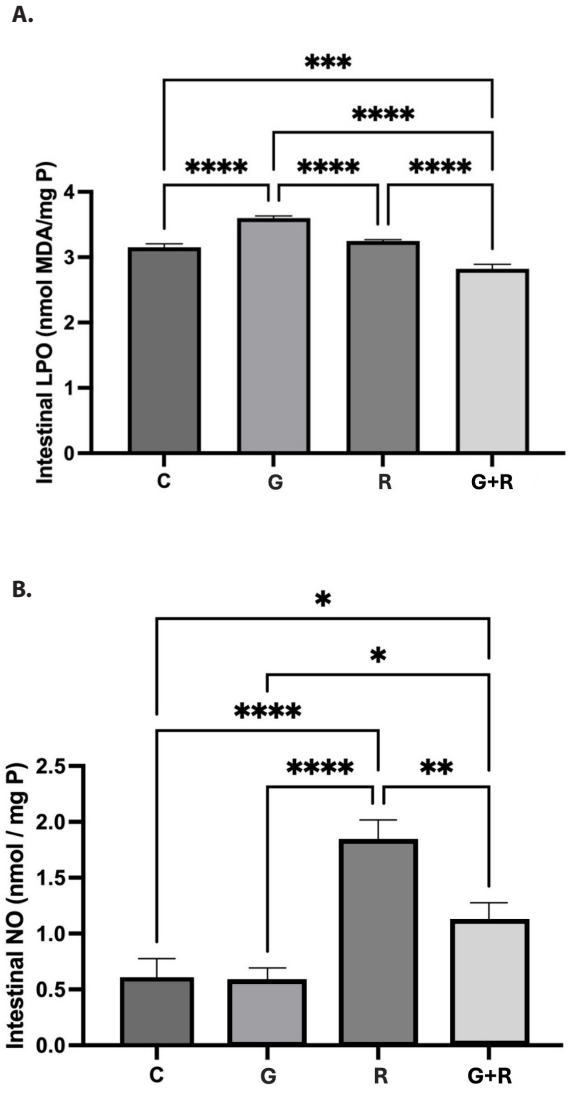


Figure 1. a) Lipid peroxidation (LPO) levels of the groups. b) Nitric oxide (NO) levels of the groups. Data are presented as mean ± SD; **** p<0.0001, *** p<0.001, ** p<0.01, *p<0.05. C: control group, G: gingipain-injected group, R: rotenone-exposed group, G + R: gingipain-injected and rotenone-exposed groups.

During the injection process, zebrafish were anaesthetized via rapid cooling for 10 s. Subsequently, the fish were positioned ventral side up in a groove on a wet sponge, and the related agent was injected intraperitoneally using a Hamilton injector. Water tanks and exposure solutions were refreshed every 48 h. After 4 weeks, the fish were sedated through rapid cold exposure, and euthanized by decapitation. Intestinal samples were obtained, and stored at -20 °C for future analyses. The biochemical and data analyses were performed by blinded researchers.

Biochemical Analyses

Zebrafish intestinal tissues were homogenized to create 10% homogenates. After following centrifugation, the resulted supernatant was isolated, and prepared for biochemical analysis. The Lowry et al. approach was used to measure total protein levels, and the outcomes were expressed as a Unit per protein (13). A byproduct of lipid peroxidation (LPO), malondialdehyde levels, were assessed using the technique described by Yagi (14). Quantities of nitric oxide (NO) were assessed by the approach of Miranda et al. (15). Superoxide dismutase (SOD) activity was evaluated using a photo-oxidation reaction (16). The Habig & Jacoby technique was used to measure glutathione-S-transferase (GST) enzyme activity at 340 nm (17). The activity of acetylcholinesterase (AChE) was determined by following Ellman et al.'s protocol (18).

Statistical Analyses

Statistical power analysis was used to establish the sample size, with an emphasis on identifying small effects. GraphPad Prism 9.0 software (GraphPad Software, United States) was utilised for statistical analysis, and the outcomes were presented as the mean ± standard deviation. Dunn's multiple comparison tests were used after the Kruskal-Wallis test to compare the data. A p-value of less than 0.05 ($p<0.05$) was selected for determining statistical significance.

RESULTS

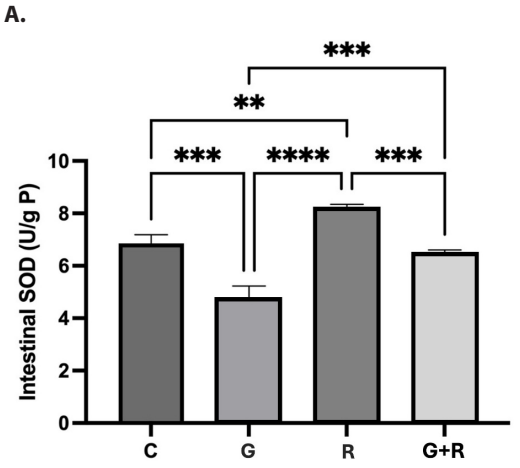
Results of the Biochemical Analyses

Gingipain injection significantly increased intestinal LPO levels compared with the C, R, and G + R groups. In comparison with the G, R, and control groups, the LPO levels of the G + R group were significantly decreased (Figure 1a).

The levels of NO in the R and G + R groups were significantly elevated compared with those in the control group. When compared with the G group, the NO levels in the G + R group were significantly higher, and when compared with the R group, the levels were significantly reduced (Figure 1b).

The R group's intestinal GST activity was significantly higher than that of the control, G, and G + R groups. The GST activity of the G and R groups differed significantly (Figure 2b).

SOD activity was significantly reduced in the G group and significantly elevated in the R group compared with that in the C group. In comparison to the G group, SOD activity was significantly increased in the R and G + R groups. The G + R group's SOD activity was much lower than that of the R group (Figure 2a).



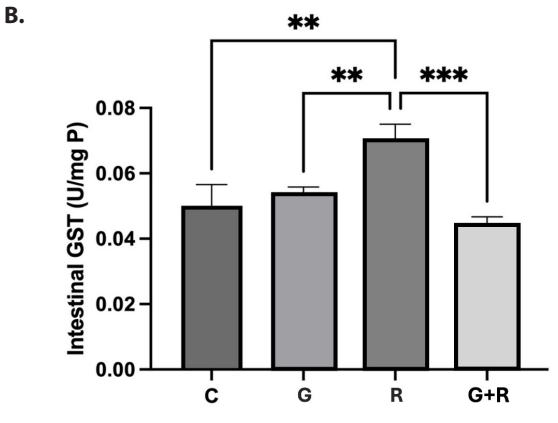


Figure 2. a) Superoxide dismutase (SOD) activity of the groups. b) Glutathione-S-transferase (GST) activity of the groups. Data are presented as mean ± SD; **** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05. C: control group, G: gingipain-injected group, R: rotenone-exposed group, G + R: gingipain-injected and rotenone-exposed groups.

AChE activities were significantly increased in the R and G + R groups compared with the control group. The AChE activity of the G group was significantly decreased compared with the control, R, and G + R groups (Figure 3).

DISCUSSION

Owing to the interconnected nature of the oral cavity and intestinal tract, numerous periodontopathogenic bacteria can migrate to the intestines through swallowing and contribute to dysbiosis in gut microbiota. In the literature, various animal studies have shown that oral *P. gingivalis* administration impairs intestinal permeability, causes dysbiosis, and intensifies inflammation in the intestines (19). The pathogenicity of *P. gingivalis* is significantly organized by gingipain proteases, which have the most attraction among the virulence factors of the bacterium. *P. gingivalis* and gingipain are also linked with the progression of neuroinflammation (20), and inhibition of gingipain decreases the severity of the inflammatory state (3).

Continuous dysbiosis in the gut microbiome leads to alterations in ENS signaling and can affect the CNS, potentially acting as a trigger for neuroinflammation. This interconnected relationship between the oral cavity, intestines, and brain is called the "oral-gut-brain axis" and is a recent growing area of interest (21). In our study, we demonstrated the impact of the endotoxin gingipain on the activity of AChE and the oxidant-antioxidant status in the gut tissues of rotenone-exposed zebrafish.

ACh is a primary neurotransmitter essential for regulating the cholinergic system in the CNS and ENS. In the intestines, ACh is essential for the functioning of digestive processes, including

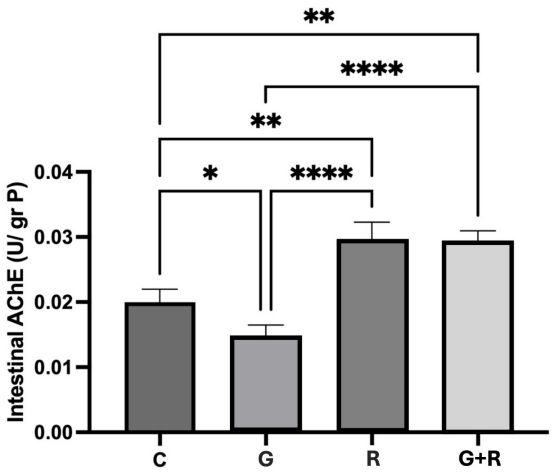


Figure 3. Acetylcholinesterase (AChE) activity in the groups. Data are presented as mean ± SD; **** p<0.0001, *** p<0.001, ** p<0.01, * p < 0.05. C: control group, G: gingipain-injected group, R: rotenone-exposed group, G + R: gingipain-injected and rotenone-exposed groups.

the contraction of smooth muscles and secretion of digestive enzymes (8). AChE is an enzyme that regulates the levels of ACh, ensuring that the signaling is properly terminated. The intricate balance between ACh and AChE activity in the gut is important for the proper functioning of the digestive system, and any disruption in this balance can lead to gastrointestinal problems. In our study, gingipain injection significantly decreased AChE activity compared with the control group, proposing a dysregulated increase in ACh levels. Although ACh is an essential regulator of the physiological functions of the gut, excessive elevations in ACh levels could lead to overstimulation of the smooth muscles of the intestines, hypersecretion of digestive enzymes, increased motility, spasms, and disrupted nutrient absorption. In addition, gastrointestinal disorders such as IBS may involve imbalances in neurotransmitter levels, including ACh (9). Our results demonstrated that gingipain can affect the cholinergic system by dysregulating AChE activity, potentially causing improper functioning in the ENS. This impairment may affect the CNS via the gut-brain axis and may potentially cause neuroinflammation.

Rotenone is a strong neurotoxin that can penetrate the blood-brain barrier and accumulate in the mitochondria of dopaminergic neurons. This accumulation inhibits mitochondrial complex I activity, triggering an increased generation of reactive ROS, contributing to dopaminergic neurone degeneration and inducing parkinsonism in zebrafish models (10). The rotenone concentration utilized in our research was based on the study of Unal et al. (22). We detected a substantial increase in AChE activity after 4 weeks of rotenone exposure in both the R and G + R groups compared with the control group. This increase underline a notable decrease in ACh activity associated with reduced intestinal motility and constipation (23). These findings align with research showing a higher incidence of IBS and constipation in individuals with PD than in healthy controls (24). Our results highlight the potential relevance of gastrointestinal dysfunction in PD.

Under conditions of elevated oxidative stress, the excessive generation of ROS interacts with cell membrane lipids, causing their oxidation and disrupting normal cellular function (25). High levels of oxidative stress in intestinal cells can cause damage to intestinal epithelial cells and the gut mucosal barrier. This damage sets off an inflammatory response that releases proinflammatory cytokines and the influx of immune cells to the area. Chronic inflammation in the gut contributes to the pathogenesis of gastrointestinal disorders and neurodegenerative diseases through the gut-brain axis (25). In this study, 4 weeks of gingipain injection remarkably increased LPO levels in the zebrafish intestines compared with the control group, indicating a major disruption in the oxidant-antioxidant balance. In a previous study by our research group, where we injected a single dose of gingipain into zebrafish, and evaluated the LPO levels in gut tissues after 6 h, the antioxidant activity of GST was sufficient to detoxify the LPO levels (26). In the present study, we demonstrated that 4 weeks of chronic gingipain administration intensified intestinal cell damage by further enhancing the oxidation of cell membrane lipids. This elevated oxidative stress can deteriorate the intestinal barrier, trigger inflammation, and compromise ENS signaling, leading to neuroinflammation/neurodegeneration via the gut-brain axis. In our study, there was no significant difference in LPO levels when comparing the R group with the control group. This result is possibly due to the counterbalancing of LPO levels, facilitated by the significantly elevated antioxidant enzyme levels, SOD, and GST. According to our findings, LPO levels were reduced in the G+R group compared with the G and control groups. This result may indicate that the oxidative response to gingipain in the gut was compromised in rotenone-exposed zebrafish.

SOD and GST both play important roles in distinct detoxification pathways in cell metabolism. SOD is an enzyme that catalyzes the degradation of highly toxic superoxide (O_2^-) molecules into oxygen, water, and hydrogen peroxide, preventing the formation of highly reactive and harmful ROS. GST is an antioxidant enzyme that catalyzes the conjugation of reduced glutathione, xenobiotics, and endogenous compounds with glutathione, facilitating their detoxification and removal from the cell. Dysfunction in antioxidant function in cells has been linked to various diseases associated with oxidative stress, including neurodegenerative diseases (27). In our study, rotenone exposure significantly increased the SOD and GST activities compared with the control for balancing the oxidative stress caused by the neurotoxin. Gingipain injection caused a significant reduction in the SOD activity compared with the control group, which may be due to the consumption of the SOD enzyme to detoxify elevated oxidative stress. Injection of gingipain in the R group also resulted in a significant decrease in both SOD and GST levels. In reaction to increased oxidative stress by rotenone exposure, although the activities of antioxidant enzymes (GST and SOD) were initially upregulated, the cumulative effects of prolonged inflammation generated by gingipain and continuously elevating oxidative stress may overwhelm the cellular defence mechanisms leading to a subsequent decline in the activities of antioxidant enzymes.

NO is involved in various physiological functions within the gastrointestinal system. It serves as a regulator of smooth muscle tone, has antimicrobial properties, contributes to the immune defense in the gut, and controls microbial balance. Although NO is not a direct marker of oxidative stress, under elevated conditions, it can react with ROS and contribute to oxidative damage (28). It is also important in the functioning of ENS, and dysregulation of NO has been observed in individuals with PD (7). In our study, rotenone exposure significantly elevated NO levels compared with the control group since it is a strong neurotoxin that creates high levels of oxidative stress. In the G+R group, there was a significant reduction in NO levels compared with the R group. High levels of ROS can reduce the bioavailability of NO by directly reacting with it, and forming peroxynitrite ($ONOO^-$), and it can impair the function of nitric oxide synthase (NOS), which is involved in NO production (29). In addition, a healthy gut microbiota contributes to the

production of NO, and an imbalance in the microbiome may negatively impact its synthesis (30). Therefore, in our study, the combination of the bacterial endotoxin gingipain and PD-inducing neurotoxin may compromise NO production in the gut, potentially leading to an imbalance in the regulation of ENS.

In our study, we studied the effects of gingipain, the most destructive virulence factor of *P. gingivalis*, on the zebrafish intestine, which may be important because of its potential impact on PD via the gut-brain axis. Gingipain caused significant imbalances in oxidative status and dysregulated AChE activity. The cumulative effect of the endotoxin gingipain and neurotoxin rotenone resulted in pronounced disruptions in oxidant-antioxidant balance, suggesting a potential compromise in cellular defense mechanisms. Moreover, to the best of our knowledge, this is the first study to show the impact of chronic gingipain exposure on AChE activity and oxidative status in the gut tissues of rotenone-exposed zebrafish. Our research highlights the link between an endotoxin produced by pathological periodontal microbiota and gut dysbiosis, which may have an effect on neurodegenerative diseases, particularly PD, underlining the important role of regular periodontal check-ups to keep the periodontal tissues healthy for maintaining a healthy ENS and CNS. Our findings will guide future research on the endotoxins of periodontal pathogenic bacteria and their connexion to neurodegenerative diseases in conjunction with the oral-gut-brain axis.

Ethic Committee Approval: Approval was received for this study from Marmara University Animal Experiments Local Ethics Committee. Protocol code: 17.2022mar.

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Author Contributions: Conception/Design of Study- I.I., M.B., D.C., E.E.A., K.N.K.; Consultant: I.I., M.B., I.U., D.C.; Experiments: I.I, E.E.A., K.N.K.; Drafting Manuscript- I.I., M.B., I.U., D.C.; Critical Revision of Manuscript- E.E.A., K.N.K.; Final Approval and Accountability- I.I., M.B., D.C., E.E.A., K.N.K.

Conflicts of Interests: The authors declare that they have no competing interests.

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