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Effect of Chronic Unpredictable Mild Stress on Hippocampus and Serum Markers in Rats

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ABSTRACT

Objective: Unpredictable stress is a common factor that we encounter in our daily lives. In this context, we wanted to investigate the morphological effects of a chronic unpredictable mild stress (CUMS) model on rat hippocampus and serum levels of brain derived neurotrophic factor (BDNF) and glial fibrillary acidic protein (GFAP).

Materials and Methods: This study was carried out on 16 Wistar albino rats which were divided into control and experiment groups. The CUMS model protocol was applied, and the morphological structures of the hippocampus were examined. The serum GFAP/BDNF levels were measured by ELISA method.

Results: The mean BDNF level was 1.65 ± 0.17 ng/mL in the control group and 2.25 ± 0.29 ng/mL in the CUMS group. The mean GFAP level was 3.04 ± 0.45 ng/mL in the control group and 2.96 ± 0.58 ng/mL in the CUMS group. When the polymorph cell layer (PCL) in gyrus dentatus was examined; the mean number of cells in the control group was 20 ± 1.38 for the left PCL and 17 ± 1.35 for the right PCL. In the stress group; the mean number of cells in the polymorph cell layer of gyrus dentatus was 27 ± 2.08 cells for the left side and 23 ± 2.01 for the right side.

Conclusions: As a result, morphological changes were observed in the gyrus dentatus region which has an important role in memory formation. The cells in the polymorph cell layer of gyrus dentatus underwent changes under stress.

Keywords: Chronic stress, hippocampus, brain derived neurotrophic factor (BDNF), glial fibrillary acidic protein (GFAP)

INTRODUCTION

Homeostasis is the conservation of the organism's cellular balance against environmental factors (1). Stress is considered as a factor that disrupts homeostasis (2). In stressful situations, there are changes in the levels of many biomediators and at the same time, stress factors cause changes in various reflex communication pathways. These changes can cause morphological alterations in tissues that are highly sensitive to external factors such as the brain. The imbalance of these changes causes pathologies in living organisms (3).

The hippocampus area is related to the cognitive process of the brain such as memory and sense of direction in mammals (4). Gyrus dentatus is a morphological and functional neuroanatomic region of the hippocampus that consists of three basic morphological layers curled into each other in rats. These layers are molecular, pyramidal and polymorphic cell layers. Degeneration of the cells in these layers plays a part in the etiopathology of many neurological diseases (5).

Chronic stress conditions cause adaptations to regain homeostasis in the organism. These adaptations are biological steps that regulate the response to stress (6).

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Intermediary biological factors released from these pathways enable the rearrangement of many biological structures and adaptation of the physiological structure of the organism to the stress condition (2). If stress factors are at a level that disrupts cellular homeostasis and the adaptation process is insufficient, tissue and organ pathologies emerge. In particular, chronic stress may have irreversible consequences for the structural and functional integrity of the cell.

Neurons are in constant interaction with the microenvironment and are very sensitive to external stimuli. If the level of stress in which an organism interacts increases, disturbances in cellular biological pathways may occur (7). The adaptation mechanisms of the organism undergo reorganization to balance this situation (8). Neurons are surrounded by astrocytes and play an active role in many metabolic activities. Disturbances that may occur in astrocytes are directly related to neuron survival. Glial fibrillary acidic protein (GFAP) is an intermediary filament produced by the astrocyte cell throughout the central nervous system (CNS). In addition, an increased GFAP level is used as a marker for reactive astrogliosis pathology in animal models (9).

Neurotrophin is a fundamental signaling molecule in the CNS responsible for neuron growth, axon elongation, and the existence of a synapse during the developmental period. Also, the brain-derived neurotrophic factor (BDNF) protein is the most extensive growth factor in the CNS which is essential for neuronal survival (10). Along with CNS effects; BDNF, a neurotrophic peptide, also has peripheral effects and these effects are related to regulating the peripheral energy metabolism. This reasonably effective neurotrophic factor is being studied as a possible marker for the nervous system in mammals (11).

In this context, it was intended to investigate the morphological effects of chronic mild stress on hippocampal tissue and serum BDNF/GFAP levels in rats.

MATERIALS AND METHODS

Animals and Standard Procedures

In the study, four-months-old 190-200 gr female *Wistar albino* rats (n=16) were used. We divided the rats into two groups in standard cages two weeks before the initiation of the study process and provided standard room conditions. Menstrual cycles of rodents were considered and analyzed. During anesthesia; a combination of ketamine hydrochloride (90 mg/kg; Ketalar, Parke-Davis) and xylazine hydrochloride (12mg / kg, 2%; Rompun, Bayer) was administered intraperitoneally to the rodents. Euthanasia performed by cervical dislocation under anesthesia. Afterwards, intracardiac blood collection was performed. The principles of "Guidelines for the Care and Use of Laboratory Animals" were applied.

Stress Model and Groups

The chronic unpredictable mild stress (CUMS) method was chosen as a stress protocol because it prevents rodents from learning stress factors and is a reliable method that has been used in many studies. In this study, the CUMS protocol which was previously defined by Willner P. (12) was used as a stress model. A total of eight different stressors were identified (Table 1) and the order of the stressors were previously determined. At the end of the experiment, anhedonia behaviors which are considered as an indicator of the depression status of the animals were closely monitored and measured.

Table 1. Stressors applied in CUMS model.

1. Cage inclination, 45°C / 24 hours	5. Changing day-night cycle
2. Hanging from the tail, 1 minute	6. Cage shaking, 10 minutes
3. Buoyancy in cold water 4°C / 5 minutes	7. Cage wetting 200 mL / 24 hours
3. Buoyancy in hot water 45°C / 5 minutes	8. Exchanging sawdust between cages

In this study, two groups were formed as the experimental group and control group. Then we applied the stressors to the experimental group for 28 days. The order of the application protocol was randomly determined before the start of the experiments.

Sucrose Consumption Test

During the 4th week of the experiment, a sucrose preference test was conducted to measure anhedonic behaviors in stressed animals. For the test, two water bottles were placed in each cage and applied for six days. One bottle contained 200mL of 20% sucrose solution, and the other contained 200mL of tap water. The rats were allowed to drink from both bottles during the first five days for habituation. The water bottles were changed every 12 hours. Sucrose consumption was calculated as the ratio of sucrose consumption to total consumption: Sucrose consumption = (sucrose consumption x 100) / total consumption.

Histological Preparation and Procedure

Brain tissues were fixed in 10% buffered formaldehyde for a week. Histological preparations were performed as previously described (13). Brain tissues were dehydrated through upgrading levels of ethanol solutions and cleared in xylene then embedded in paraffin. Before proceeding to the histological serial sectioning stage, the coordinates of the desired cross-sectional area were determined according to the region of bregma (Bregma -3.00 mm) using the brain atlas (*Paxinos, Watson*) of the hippocampus region of the animals whose brain tissues were completely removed. Sections of the brain tissues were stained with hematoxylin and eosin to determine the hippocampus microstructure. The end boundary of the pyramidal cell layer in the CA3 region within

the hippocampus tissue was determined as the counting area and a border perpendicular to the tissue axis was drawn at this point. Cell count was performed in the region between this axis and the granular cell layer of the hippocampus.

ELISA Method and Biochemical Procedures

Blood samples (3mL) were obtained from the left ventricle. Blood specimens were allowed to clot for two hours at room temperature and centrifuged for 15 minutes at 1000 g force at 4-8^{0}C. The supernatants were collected and diluted 1/10 before the assay. Serum samples were stored at -40°C. The serum BDNF and GFAP levels were determined with Enzyme Linked Immunosorbent Assay (ELISA; Elabscience USA) kits were used and measured with the Alisei Quality System Seac Radim Company analyzer (Italy/Rome)-ELISA reader on the basis of the manufacturer's instructions (BDNF, Catalog number E-ELR1235; GFAP, Catalog number E-ELR1428). The results were multiplied by the dilution coefficient and their concentrations were calculated according to the kit standards.

Data Analysis and Statistics

A Kolmogorov-Smirnov analysis was performed for the normal distribution suitability test in the statistical evaluation of the study results. An independent t-test was applied for the values that corresponded to normal distribution. The Mann Whitney U test was used for the parameters that did not comply with normal distribution. The p values of 0.05 or less were considered statistically significant. The statistical analysis SPSS 22.0 (IBM, Packet program) was used. The GraphPad Prism8 package program was used for graphics design.

RESULTS

During the study, animal weights were monitored, and the mean weights at the beginning of the study were 193 gr for the control group and 198 gr for the stress group. Body mass increase was less in rats under chronic stress protocol when compared to the control group. Even so there was no significant difference between the mean weights of the groups. The temporal change graph of the mean values of animal weights according to the groups is given in Figure 1.

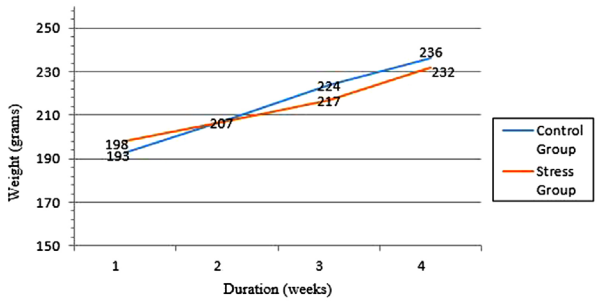

Figure 1. Variations in the average weight of animals according to the groups and time.

The sucrose consumption test, which is an important parameter in stress model validation, was applied to the rodents. In the study, there was a significant decrease in sucrose consumption when the rats underwent the CUMS procedure as compared to the control group ($p< 0.05$).

We performed a histological evaluation of the hippocampus tissue separately for the right and left sides. We measured the hippocampus thickness for both groups on the vertical axis 'coronal section' and compared them statistically. The molecular, pyramidal and polymorphic area thicknesses with complete hippocampus tissue thicknesses and subdomains were also evaluated among the groups. The mean thickness of the left hippocampus was 1,581 ± 61.01 µm for the control group. In the stress group rats, the mean left hippocampus thickness was 1,486 ± 48.94 µm. The mean thickness of the right hippocampus was 1,544 ± 36.75 µm for the control group. In the stress group rats, the mean right hippocampus thickness was 1,478 ± 91.82 µm. Figure 2 shows the right and left hippocampal thicknesses according to the groups.

When the hippocampus thicknesses of the rats in the experimental and control groups were compared statistically, no significant difference was found in terms of their thickness ($p>0.05$). Between the groups; no significant difference was found between molecular, pyramidal and polymorphic area thicknesses ($p<0.05$). When the polymorphic cell layer (PCL) in the gyrus dentatus was examined, the average number of cells was 20 ± 1.38 cells/field for the left PCL and 17 ± 1.35 cells/field for the right PCL in the control group. In the stress group, the average number of cells in the polymorphic cell layer of the gyrus dentatus was 27 ± 2.08 cells/field for the left side and 23 ± 2.01 cells/field for the right side. The cell count in the polymorph cell layer of gyrus dentatus of the hippocampus is given in Figure 3.

When the gyrus dentatus regions were examined, there was a statistically significant difference among the groups cell count in the PCL ($p<0.05$). When the stress group and control group were compared for the left hippocampus, there was a significant increase in the cell count in the relevant area of the stress group compared to the control ($p=0.021$). A statistically significant increase in the cell count in the dentate gyrus of the rodent brain in the stress group was found as compared to the control for the right hippocampus ($p=0.026$). When both experimental groups were compared statistically, there were no significant differences between cell counts in the gyrus dentatus regions of the right and left hemispheres ($p>0.05$).

The obtained histological images were examined morphologically in both groups. It was observed that the cells in the polymorph cell layer of the animals belonging to the stress group were morphologically spindled. Furthermore, there was a statistically significant increase in the polymorph cell count in the stress group ($p<0.05$). In the PCLs of the rodents belonging to the stress group, the cells increased with a spindle morphological structure. The histological images of the stress and control groups are shown in Figure 4.

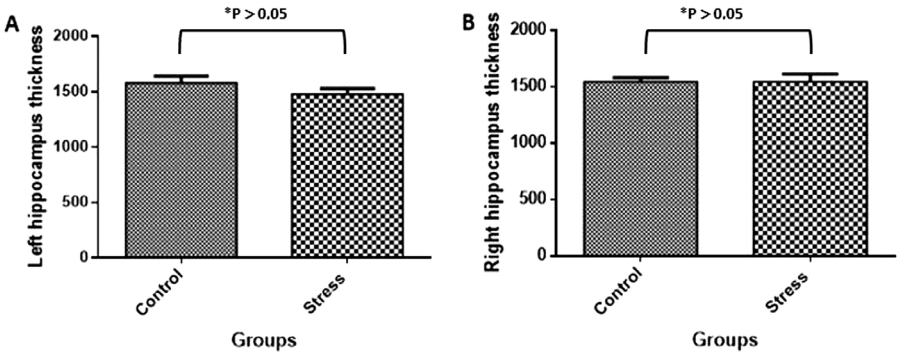

Figure 2. Right and left hippocampus thicknesses for groups. A. Inter-group hippocampal thickness for left hippocampus coronal section, B. Inter-group hippocampal thickness for right hippocampus coronal section.

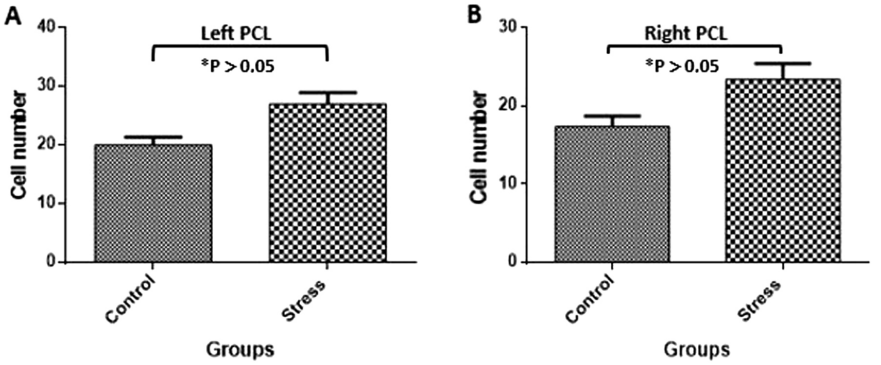

Figure 3. Cell count in the polymorph cell layer, gyrus dentatus. A. Inter-group cell count for the left PCL in gyrus dentatus, B. Inter-group cell count for the right PCL in gyrus dentatus.

We performed the BDNF and GFAP measurements via the ELISA method in serum obtained after intracardiac blood collection. The mean BDNF level was 1.65 ng/mL in the control group and 2.25 ng/mL in the CUMS group. The mean GFAP level was 3.04 ng/mL in the control group and 2.96 ng/mL in the CUMS group. There was no statistically significant difference among the groups in terms of the BDNF and GFAP serum levels ($p>0.05$, Figure 5).

DISCUSSION

Stress has a significant impact on the brain, and it can cause changes in brain function and structure. The response of stress hormones affects various brain regions, particularly the hippocampus, which is crucial for memory formation. The hypothalamic-pituitary-adrenal (HPA) axis is the primary biological pathway for regulating the effects of stress on the organism. Stress hormones which a steroid structure secreted through this system makes radical changes in the metabolism (3). A thorough understanding of the mechanism of the action of stress can be an effective way to treat diseases such as neuron degeneration.

It is known that stress affects neurobiological pathways and in this way affects neurons, astrocytes and microglias (14). During this process; neurons, astrocytes and glial cells are affected by the HPA axis by means of immunological pathways, and neurotrophic factors. One of the most characterized neurotrophic factors is BDNF, which modulates brain health and neuronal survival (11). The activity dependent expression of BDNF is a crucial mechanism for synaptic plasticity that improves a mammals' learning ability (15). This study investigated whether there was a correlation between BDNF, which has important effects on the biological pathways of CNS and the morphology of the hippocampus under stress. In this study, no difference was found between the experimental group of rats BDNF serum levels which had been applied with the CUMS. The GFAP is a structural filament protein that is mainly expressed in astrocytes (9). The astrocytic cytoskeleton filament protein GFAP plays a role in many processes in the CNS. It has important effects, especially on neuronal survival. Astrocytes have critical roles in maintaining neuronal metabolism, regulating neuroimmunological reactions and maintaining the blood-brain barrier. GFAP, an astrocyte marker,

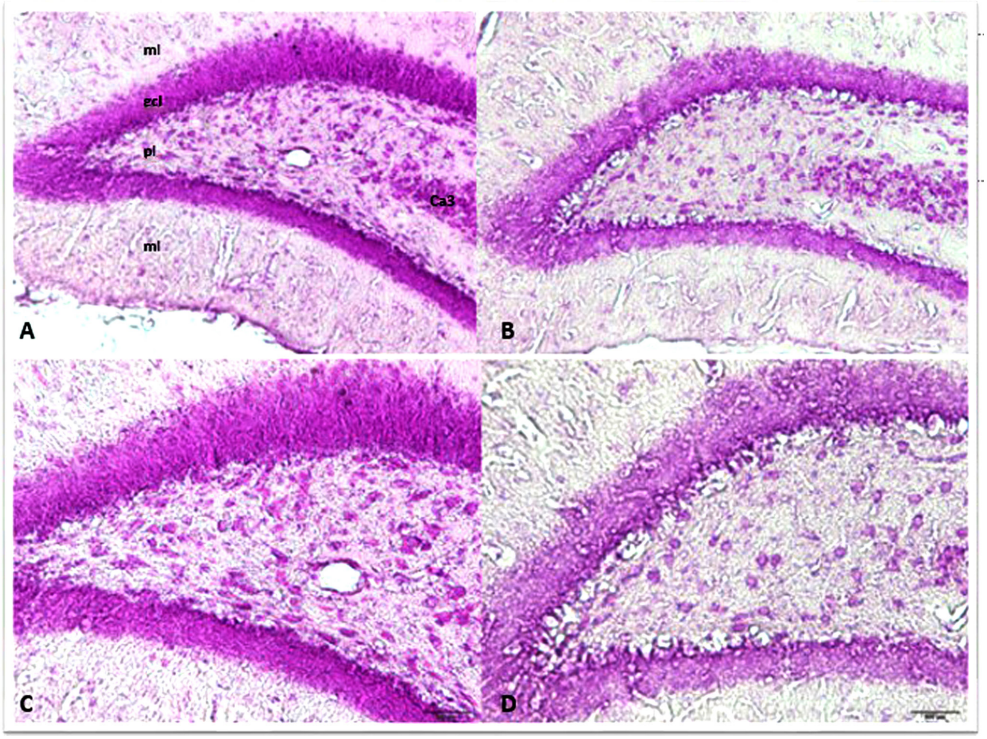

Figure 4. Microscopic images of gyrus dentatus and subunits of CUMS and control group. A. CUMS group gyrus dentatus, 200X; B. Control group gyrus dentatus, 200X; C. CUMS group gyrus dentatus, 400X; D. Control group gyrus dentatus, 400X; pl: Polymorph layer; gcl: Granular cell layer; ml: Molecular layer; Ca3: Cornu ammonis3.

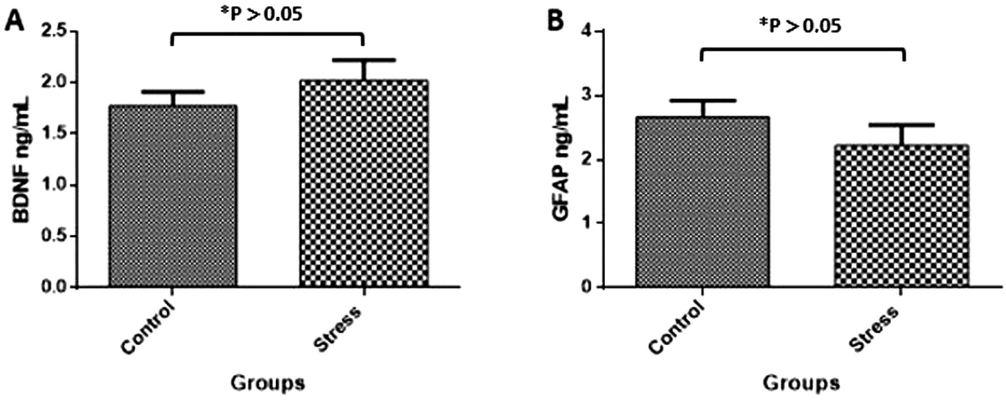

Figure 5. Serum BDNF and GFAP levels for CUMS and control groups. A. Intergroup comparisons of serum BDNF levels, B. Intergroup comparisons of serum GFAP levels.

increases expression in a brain injury or CNS degenerations (16). In this context; we examined the GFAP serum levels as a biomarker for astrocytes, an important component of CNS, as well as hippocampus morphology. There were no significant differences in the GFAP serum levels among the groups.

Excessive stress is a phenomenon that damages the brain tissue. The hippocampus is damaged both cognitively and morphologically under this condition (6). It is even reported that there is a decrease in hippocampal volume with chronic depression. This volumetric reduction was reported to be due to the pyramidal cell depletion in the cornu ammonis region (17). The organism activates an adaptation mechanism in response to stress. However, the hippocampus also degenerates during chronic stress conditions that disrupt hemostasis (10). The most devastating outcomes occur during chronic severe

stress situations where the adaptation mechanism of the cell is inadequate. Cellular degeneration is inevitable in prolonged periods of intense stress (18). However, it should be noted that mild stress provides cellular stimulation and keeps the organism alert (19). It is reported that neurogenesis decreases during chronic severe stress conditions and increases in acute stress conditions in the hippocampus area. *In vivo* studies showed that the hippocampus also increased neurogenesis in single dose corticosterone administration. Other studies showed that stress hormones cause an increase in fibroblast growth factor (FGF2) receptors in rats hippocampal cells (20). In this study, no difference was found between the hippocampus thickness of rats. The biological pathways underlying the different response mechanisms of animals to stress is thought to contribute to the development of treatment options for stress-dependent diseases. In this context; we applied a mild stress model that is frequently faced in daily life and used an unpredictable experimental model. We prevented the animals from getting accustomed to stress and adapting to it. We examined the morphological changes and cell count changes in the polymorph cell layer in the hilum of the dentate gyrus area under chronic unpredictable mild stress. This study observed a cellular increase in morphologically spindled cells in the region of the PCL. We determined that there was a statistically significant increase in the cell count in the PCL. The close relationship between changes in this area and memory is emphasized in the literature. Degenerations in this part of the gyrus dentatus are closely associated with memory disorders (21).

In another *in vivo* study; when the hippocampal neurogenesis of rodents was inhibited under stress conditions, temporal prolongation in the decrease of stress hormones to normal serum levels was observed. In other words, inhibited neurogenesis was associated with elevated stress-induced high glucocorticoid levels for longer periods. Hippocampal neurogenesis was suggested to buffer the stress responses and depressive behaviors in mammalians (22). It is suggested that hippocampal neurogenesis has a buffer function against stress conditions, in mice (19). In this study, we determined cellular changes between the groups 'stress/control' in the PCL of gyrus dentatus against mild stress. The cellular increase in the stress group is considered to be part of an adaptation mechanism to stress, which is also mentioned in the literature. Possible cellular increases in the polymorph cell layer under chronic unpredictable stress may bring about new cellular connections. In this case, it is thought that new cells originating from the progenitor area may cause memory disorders. Newly formed cells can create new synapses independent of the information flow and create new pathways in memory formation in this area. This process is formed as a result of the organism's struggle with stress and can create disruptions in memory condensation (21). This could provide a neurobiological approach to explaining post-stress memory disorders and contribute to improving treatment options.

CONCLUSION

This study aimed to determine how CUMS morphologically affects the hippocampus of rodents. In addition, we evaluated the serum values of BDNF and GFAP which are accepted as biological markers for neurons and astrocytes with morphological changes. When we compared the stress and control groups, we observed changes in the hippocampus of animals under stress conditions. These changes were observed in the gyrus dentatus region which has an important role for memory formation. The cells in the PCL of gyrus dentatus underwent changes under stress. In the results obtained, there was an increase in the cell count in the PCL of gyrus dentatus in the stress group. In addition, the cellular morphologies changed and became spindle shaped. However, the study did not find any significant difference when CUMS and the control groups were compared in terms of hippocampus thickness. In addition, there was no significant difference in the BDNF and GFAP serum markers levels between the groups.

Ethics Committee Approval: Ethical approval of the study was obtained from Animal Experiments Local Ethics Committee, Kocaeli University (2015/44).

Authors' Contributions: Conception/Design of Study - M.D.Y., T.C.; Data Acquisition – M.D.Y., S.K.O., E.A.; Performing experiments – M.D.Y., S.K.O., E.A.; Data Analysis/Interpretation – M.D.Y, T.C.; Statistical Analyses – M.D.Y., S.K.O., E.A.; Drafting Manuscript – M.D.Y., T.C.; Critical Revision of Manuscript – M.D.Y., T.C., S.K.O., S.C., E.A., H.M.K.; Final Approval and Accountability - M.D.Y., T.C.

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

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