Experimed 2022; 12(3): 113-8

ORIGINAL ARTICLE

DOI: 10.26650/experimed.1189898

The Effect of Whey Proteins on the Brain and Small Intestine Nitric Oxide Levels: Protein Profiles in Methotrexate-Induced Oxidative Stress

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Cite this article as: Yilmaz S, Tufan E, Goksun Sivas G, Gurel Gokmen B, Dursun E, Ozbeyli D, Sener G, Tunali-Akbay T. The effect of whey proteins on the brain and small intestine nitric oxide levels: Protein profiles in methotrexate-induced oxidative stress. Experimed 2022; 12(3): 113-8.

ABSTRACT

Objectives: The aim of this study was to determine the effects of whey proteins on methotrexate (MTX)-induced brain and small intestine damage.

Materials and Methods: 30 Sprague Dawley rats (200-300 g) were divided into four groups: Control, control + whey, MTX, and MTX+whey. MTX was administered at 20 mg/kg (single dose) intraperitoneally to the MTX group rats, and 2 mg/kg of whey protein were administered by oral gavage for 10 days to the whey groups. Lipid peroxidation, glutathione, and nitric oxide (NO) levels, as well as glutathione-S-transferase and superoxide dismutase activities were measured in the brain and small intestine. SDS-polyacrylamide gel electrophoresis of the brain and intestine tissues were also carried out.

Results: While MTX treatment caused oxidative damage in the brain and small intestine, whey protein administration ameliorated MTX-induced oxidative stress. MTX administration did not change the brain's NO level, while an increase in intestinal NO level was detected.

Conclusion: MTX induced oxidative stress in the brain and small intestine changed the protein metabolism in these tissues regardless of reduced food intake. Consecutive 10-day administration of whey proteins has shown its therapeutic effect on MTX-induced brain and small intestine oxidative damage.

Keywords: Methotrexate, whey protein, brain, small intestine, nitric oxide, oxidative stress

INTRODUCTION

As a folate anti-metabolite, methotrexate (MTX) is used to treat some malignancies and immunological disorders (1). It inhibits the mammalian dihydrofolate reductase (DHFR) enzyme, which is required to generate tetrahydrofolate (THF) from folic acid and synthesizes purines and pyrimidines for the progression of the cell cycle. The mechanism of action against the DHFR enzyme appears to be the same in both bacterial and mammalian cells, despite the different drug susceptibility between bacterial strains (2). MTX causes gastrointestinal toxicity, including nausea, diarrhea, and signs of decreased nutrient absorption, while also inducing enteritis. The inhibition of DHFR is the first step in the process of MTX-induced gastrointestinal damage and is related to DNA synthesis. The mucosa's barrier function against intravascular pathogens is therefore impaired, leading to bacterial translocation and inflammation. Oxidative damage has also been detected in human intestinal cells during MTX treatment (3, 4). Miyazono and Horie (5) revealed oxidative damage to

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Submitted: 16.10.2022 **Revision Requested:** 31.10.2022 **Last Revision Received:** 03.11.2022 **Accepted:** 16.11.2022 **Published Online:** 27.12.2022

contribute the neutrophil infiltration into inflammatory areas in the intestine and also contribute to increased transendothelial and transepithelial permeability. Disturbances at any stage of digestion might promote dysregulation in various digestive and non-digestive diseases (3). MTX also increases the oxidative stress by raising the concentration of reactive oxygen species (ROS) generation, and resulting in neurotoxicity (6). A high dose of MTX has been shown to cause acute, subacute, and chronic neurotoxicity. Acute toxicity often causes temporary impairment. Subacute and chronic toxicity are linked to alterations in the brain that may result in coma and death. Furthermore, MTX interferes with the biochemical activities of folate, homocysteine, adenosine, S-adenosylmethionine/S-adenosylhomocysteine, and biopterin, resulting in biochemical alterations related to neurological symptoms (7). MTX inhibits the NADP malic enzyme and NAD (P)-dependent dehydrogenases, thus it appears to reduce NADPH availability in cells by inhibiting the enzymes for the pentose phosphate pathway. As a result of failure of the antioxidant defense system, cells may become more vulnerable to MTX-induced oxidative damage. In addition, glutathione-S-transferase (GST), a family of phase-II detoxification enzymes, has been shown to catalyze the binding of glutathione (GSH) to a variety of electrophilic compounds, including MTX, and preventing these drugs from reaching their intended cellular targets (8). Since GSH is used to detoxify MTX and is unable to show an antioxidant effect, it may not prevent the increase of lipid peroxidation.

Various antioxidants, plant extracts, and nutrients are currently being researched for reducing or eliminating the damage caused by MTX treatment. For example, melatonin (4), retinol (9), garlic extract (10), and sodium tungstate (5) have been demonstrated to protect against MTX-induced injuries. This study was concentrated on the possible antioxidant properties of whey proteins regarding MTX-induced brain and small intestine damage. Whey proteins form about 20% of all milk proteins and can be produced after the precipitation of casein at 20 ^{0}C and a pH of 4.6. Whey proteins are mostly composed of globular proteins such as lactoferrin, b-lactoglobulin, albumin, immunoglobulins, a-lactalbumin, lactoperoxidase, protease peptones, and bioactive peptides (11). Valin, leucine, and isoleucine (branched-chain amino acids) are abundant in the whey fraction that contribute to muscle growth, and the protective role of whey protein concentrate (WPC) on the intestinal barrier has also been shown (12). *In vitro,* bovine whey products and hydrolysates exhibit antioxidant activity by chelating metals; reducing lipid peroxidation; decreasing ferric ions; scavenging radicals, hydroxyls, and superoxides; and neutralizing synthetic radicals. Whey proteins have been shown to possess a protective role against cellular oxidation and an antioxidant effect by increasing antioxidant enzymes such as glutathione peroxidase, superoxide dismutase (SOD), and catalase (in lung fibroblasts, hepatocytes, and endothelial cells) (11, 13). As a result, the present study examined the potential antioxidant mechanism of whey proteins against MTX-induced brain and small intestine damage.

MATERIALS AND METHODS

Experimental Design and Animal Testing

The Marmara University School of Medicine Animal Care and Use Committee approved the study (Protocol Number: 55.2021.mar). Sprague-Dawley (male) rats weighing between 200–300 g were housed in standard conditions and fed with a regular diet. The rats were separated into four different groups: control (C), whey-treated control (C+W), methotrexate (MTX), and whey-treated methotrexate (MTX+W) groups. The dose of MTX was decided based on the study by Aykac et al. (14). MTX injections (in physiological saline, 20 mg/kg, single dose) were continued over the next 10 days using either the saline (MTX group, $n = 8$), or whey protein (2 g/kg, oral gavage, MTX + W group, $n = 8$). Other rats received either the saline (C group, $n = 6$) or whey protein (2 g/kg, oral gavage C+W group, $n = 8$) for 10 days after receiving a single injection of saline. The rats were decapitated under ether anesthesia on day 10, and brain and small intestine tissue samples were taken.

Whey Protein

Lyophilization was used to preserve the whey protein beverage (Tazelen) that had been purchased from Kaanlar Food Industry and Trade, Turkiye. The relevant groups received 2 g/kg (oral gavage) of the lyophilized whey protein beverage dissolved in tap water. The whey protein dosage was chosen based on Shimizu et al. study (15).

Biochemical Analysis

10% small intestine and brain tissue homogenates in physiological saline were prepared using a glass homogenizer. The cooling process during homogenization was done by immersing a glass homogenizer into a beaker containing ice. Tissue homogenates were then centrifuged at 3000xg for 10 minutes. The supernatant samples were used for biochemical analysis. Malondialdehyde (MDA) levels were used as a marker of lipid peroxidation (16), SOD activity (17), GST activity (18), nitric oxide (NO) levels (19), and GSH levels (20) were also determined.

SDS-Polyacrylamide Gel Electrophoresis

The basic concept of Laemmli SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used to examine small intestine tissue and brain in terms of electrophoresis (21). The BIO-RAD mini protean precast II dual slab gel apparatus was used for SDS-PAGE (BIO-RAD, USA). For protein electrophoresis, mini PAGE gels (Any kD precast polyacrylamide gel, 8.6 6.7 cm (W L), Catalog Number: 4569033, BIO-RAD, USA) were used.

Statistical Analyses

Graphpad Prism 6.0 was used to perform the statistical analyses (GraphPad Software, San Diego, CA, USA). The results were provided in terms of means and standard deviations (SD). To compare the groups, ANOVA (post hoc Tukey test) was performed, with $p < 0.05$ being considered significant. Statistical power analysis was performed on the small intestine and brain NO levels using Faul et al.'s (22) method.

RESULTS

Brain

MTX administration significantly increased MDA levels and significantly decreased the levels of GSH, SOD and GST activities compared to the control group ($p < 0.05$). NO levels did not significantly change in the MTX group compared to the control group ($p > 0.05$; Figure 1). Whey protein administration to the MTX group significantly decreased MDA levels and significantly increased GSH levels, SOD levels and GST activity ($p < 0.05$). Brain NO levels did not significantly alter with the whey protein administration to the MTX group ($p > 0.05$). Upon administering whey proteins to the control group, no significant change occurred in MDA or GSH levels, nor in SOD and GST activity ($p > 0.05$); moreover, brain NO levels did decrease significantly ($p < 0.05$; Figure 1). When considering specific effect size, level, and sampling methods, the power analysis of the brain NO levels was found to be 0.84, which indicates an 84% possibility of disproving the null hypothesis.

Small Intestine

MTX administration significantly increased MDA and NO levels and significantly decreased GSH, SOD levels and GST activity compared to the control groups ($p < 0.05$; Figure 2). Whey protein administration to the MTX group decreased MDA and NO levels and significantly increased GSH levels, SOD levels and GST activity ($p < 0.05$). Upon administering the whey proteins to the control group, significant decreases in MDA and NO levels and significant increases in GSH levels and SOD activity occurred ($p < 0.05$), while no significant change occurred in GST levels ($p > 0.05$; Figure 2). Given a specific effect size, level, and sample size, the power analysis of the small intestine NO levels was found to be 0.86, which indicates an 86% possibility of disproving the null hypothesis.

SDS Polyacrylamide Gel Electrophoresis

Electrophoretic models of brain and small intestine tissue belonging to all groups were shown in Figure 3 and Figure 4. Some differences in the densities of the brain and small intestine protein bands were detected in the electrophoretic examination. MTX administration decreased some protein bands of brain tissue detected between 50 kDa and 37 kDa, while whey protein administration to the MTX group increased these decreased bands. The majority of small intestine proteins were located at about 14, 30, 66 and 70 kDa during the administration. MTX administration decreased the density of 14 kDa and 30 kDa protein bands, while whey protein administration was able to increase these bands. Whey protein administration to the control group also increased the small intestine 30 kDa protein.

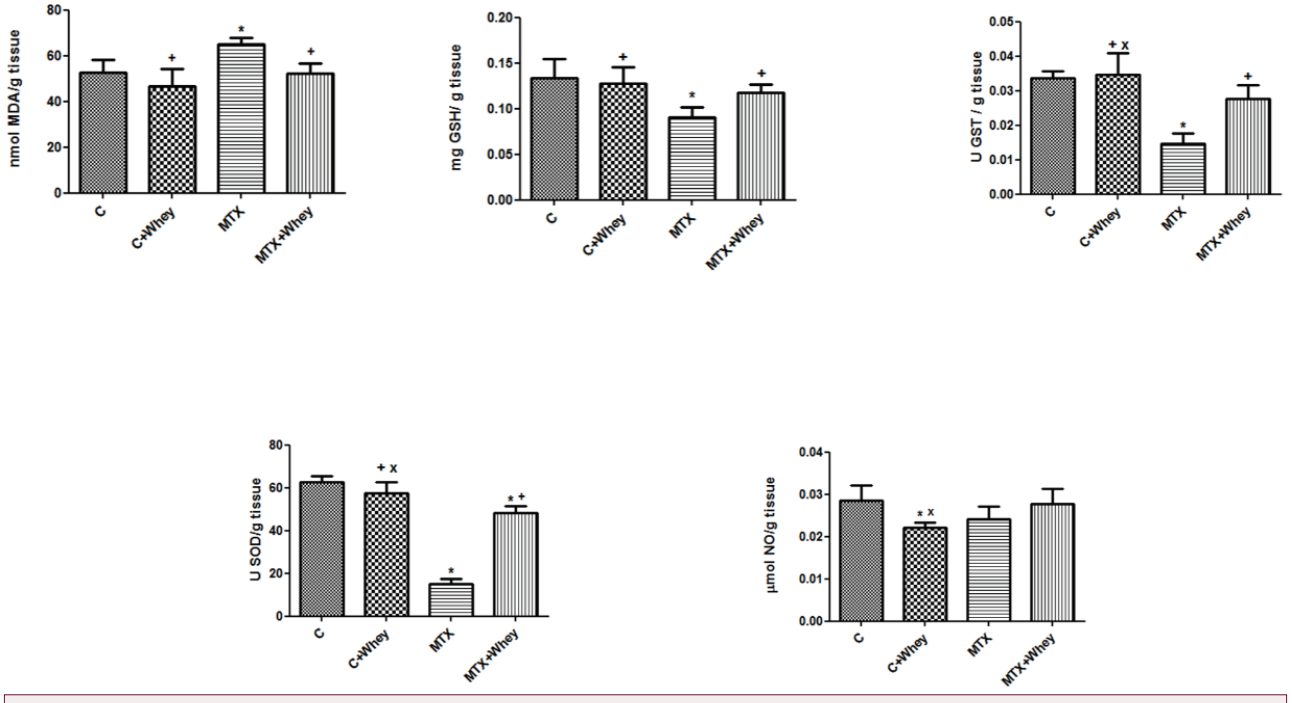

Figure 1. Biochemical analysis of brain tissue.

C: Control group, C+whey: Whey-administered control group, MTX: Methotrexate-administered group, MTX+whey: Methotrexate and whey protein -administered group, MDA: Malondialdehyde, GSH: Glutathione, SOD: Superoxide dismutase, GST: Glutathione-S-transferase, NO:Nitric oxide, *p<0.05 compared to the control group, ˣp<0.05 compared to the MTX group, ⁺p<0.05 compared to the C+whey group (n= 8).

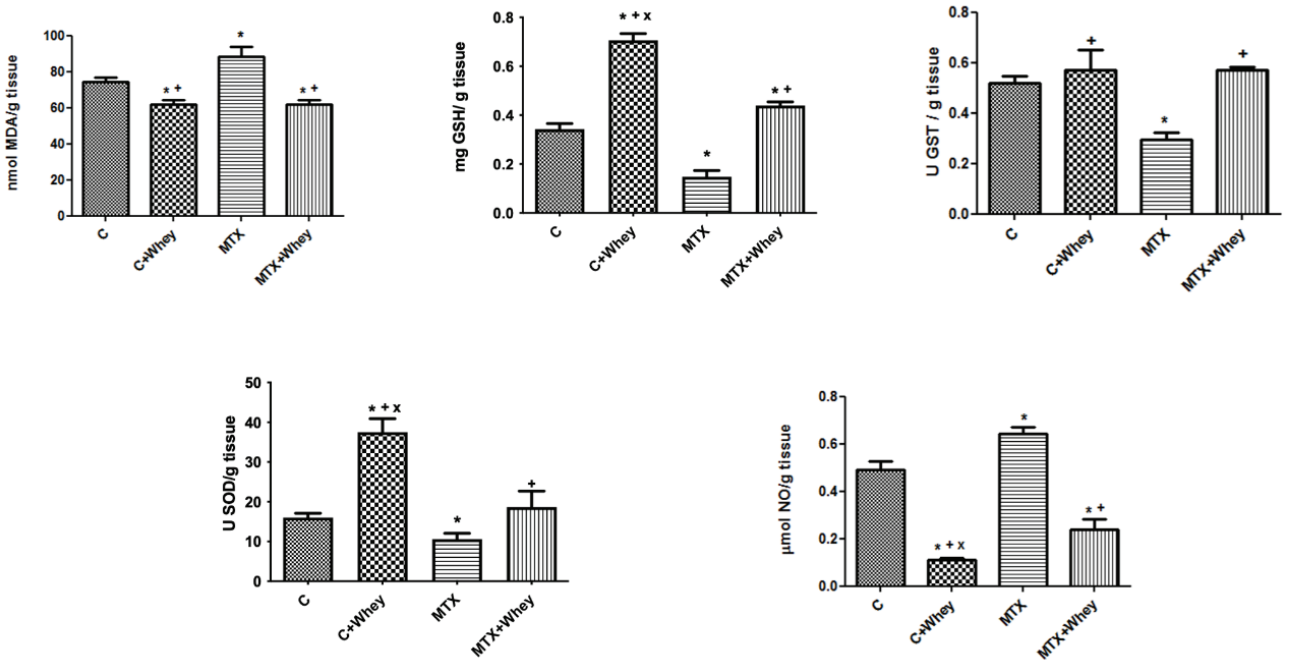

Figure 2. Biochemical analysis of small intestine tissue.

C: Control group, C+whey: Whey-administered control group, MTX: Methotrexate-administered group, MTX+whey: Methotrexate and whey protein -administered group, MDA: Malondialdehyde, GSH: Glutathione, SOD: Superoxide dismutase; GST: Glutathione-S-transferase, NO: Nitric oxide, *p<0.05 compared to the control group, ˣp<0.05 compared to the MTX group, ⁺p<0.05 compared to the C+whey group (n=8).

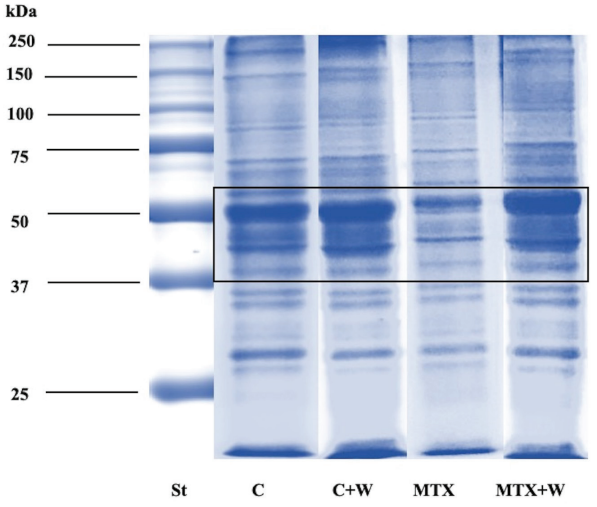

Figure 3. SDS- polyacrylamide gel electrophoresis of brain tissue.

St: Standard, C: Control group, C+W: Whey protein-administered control group, MTX: Methotrexate-administered group, MTX+W: Methotrexate and whey protein-administered group.

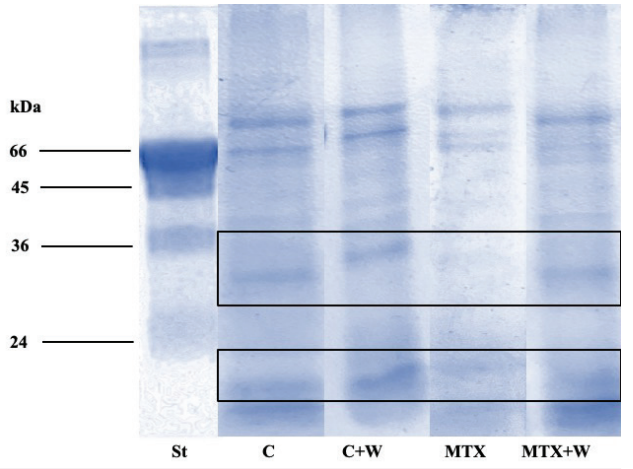

Figure 4. SDS- polyacrylamide gel electrophoresis of small intestine tissue.

St: Standard, C: Control group, C+W: Whey protein-administered control group, MTX: Methotrexate-administered group, MTX+W: Methotrexate and whey protein-administered group.

DISCUSSION

The gut-brain pathway is a network that links the central nervous system with the gastrointestinal tract and is made up of a half billion neurons that innervate the gut and immune cells in gastrointestinal circulation (23). Any damage to the brain or small intestine might disrupt this connection and affect tissues. MTX treatment can also affect intestinal microbiota growth at physiologic concentrations (24).

Oxidative stress is one of the most important pathogenic processes for neurological disorders such as Parkinson's and Alzheimer's, as well as acute conditions like stroke and brain trauma. Meanwhile, brain lesions of different etiologies change gut characteristics and microbiota (2). These new concepts may lead the way to novel treatment methods for a variety of neurological diseases. This study has shown that MTX increased the oxidative stress in both the brain and the small intestine, as well as it lowers the activity of antioxidant enzymes. GST has been found to catalyze the conjugation of GSH to some electrophilic anticancer drugs, preventing drugs from reaching their cellular targets. One reason for this to use as an antioxidant is to reduce the increased lipid peroxidation due to the application of MTX, while another reason may be that GSH binds to MTX at which point it gets consumed; namely, GSH begins a detoxification reaction. MTX did not affect the brain NO levels, whereas the small intestine NO level was increased. The effects of MTX on the SDS/PAGE profiles of the brain and small intestinal tissues were reproducible. Whey protein administration, however, reduced oxidative stress, improved antioxidant defenses, and restored the disappeared protein bands in the brain and small intestinal tissues of rats that had been given MTX. The whey protein administration to the MTX group did not alter the brain NO levels either, although it lowered NO levels in the small intestine. Whey protein administration to the control group reduced NO levels in both brain and small intestine.

NO is a key signaling molecule that plays critical functions in the neuronal and intestinal tissues, as well as in inflammatory responses. Modifications to the biomolecules caused by oxidative stress may affect their physiological function and have serious repercussions for an organism (25). Furthermore, oxidative stress may result in the uncoupling of the endothelial nitric oxide synthase (eNOS), reducing NO synthesis/bioavailability and increasing NO-dependent oxidative stress. As a result, the prooxidative and antioxidative states of cells and tissues significantly influence NO production and bioactivity (25). Although NO is necessary for healthy gastrointestinal function, some evidence exists that an excessive amount of NO may impair the digestive system (26). An increase of NO in tissue may cause an increase in NO-dependent oxidative stress in that tissue. Our findings pointed out that MTX-induced brain oxidative damage did not affect brain NO levels. However, the increase in intestinal NO levels in parallel with the MTX-induced oxidative stress may have further increased the severity of oxidative damage. When exposing intestinal epithelial cells to high NO levels, their permeability increased. Apoptosis and intestinal secretion could both be stimulated by high levels of NO. However, NO may also prevent apoptosis and lessen inflammation by preventing NF-κB activation (27). MTX also inhibits NF-κB activity (28). This study used whey proteins to reverse these MTX-induced abnormalities to a healthy condition. Whey is a protein complex found in milk and is suggested as a food supplement with several health advantages. The biological contents of whey protein such as beta-lactoglobulin, lactoferrin, alpha-lactalbumin, immunoglobulins, and glycomacropeptide exhibit a range of immune-stimulating properties. Whey can also be used as an antihypertensive, anticancer, hypolipidemic, antibacterial, antiviral, antioxidant, and chelating agent. The principal mechanism by which whey protein is considered to exert its benefits is by intracellular conversion of the amino acid cysteine to GSH, a potent cellular antioxidant (29). Cysteine contains the sulfhydryl group, which acts as a reducing agent in the prevention of oxidative stress. GSH is more efficient as an antioxidant in its reduced form (30). In this study, whey administration to the MTX group decreased the MDA levels in the brain and small intestine tissue and increased the GSH levels as well as SOD and GST activity. While this cause no change in brain NO levels, it decreased NO levels in the small intestine tissue of the control groups. The brain had a more complex protein band profile than the small intestine, but the bands were more visible. The reason for the sharper protein band is related to the brain tissue's protein content. Although the protein bands in the small intestine were not clearly visible, the reductions in protein bands caused by MTX were demonstrated to have been restored through the administration of whey protein.

The limitation of this study is examining the effects of a single of a single dose of MTX determined in the experimental animal model. However, MTX is administered to patients in repeated doses, and the application time of whey proteins could also change in this case.

CONCLUSION

The study suggests that the whey proteins may have a therapeutic impact on the MTX-induced changes and oxidative stress in brain and small intestine tissues.

Ethics Committee Approval: This study was approved by the Clinical Research Ethics Committee of Marmara University Medical Faculty (26.05.2021-50.2021).

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study - T.T.A., G.S.; Data Acquisition - S.Y., E.T., G.G.S., B.G.G., E.D., D.O., G.S., T.T.A.; Performing experiments - S.Y., E.T., G.G.S., B.G.G., E.D., D.O., G.S., T.T.A.; Data Analysis/Interpretation - S.Y., E.T., G.G.S., B.G.G., E.D., D.O., G.S., T.T.A.; Statistical Analyses - S.Y., E.T., G.G.S., B.G.G., E.D., D.O., G.S., T.T.A.; Drafting Manuscript - S.Y., E.T., G.G.S., B.G.G., E.D., D.O., G.S., T.T.A.; Critical Revision of Manuscript - S.Y., E.T., G.G.S., B.G.G., E.D., D.O., G.S., T.T.A.; Final Approval and Accountability - S.Y., E.T., G.G.S., B.G.G., E.D., D.O., G.S., T.T.A.

Conflicts of Interest: The authors declare no conflict of interest.

Financial Disclosure: The authors declare that this study has received no financial support.

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