1. PROTOCOL INFORMATION

Title: The Effect of High Antioxidant Cacao on Behaviors in Children with Autism Spectrum Disorder
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Phase of Study: September 2017-September 2018

2. PRINCIPAL INVESTIGATOR'S INFORMATION

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3. STUDY PERSONNEL

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4. STUDY INFORMATION

The location of the research will take place at each child's individual school and in their home environment. Expected start date is August 2017 and expected end date of research is August 2018. This is an experimental repeated measures design.

5. INCLUSION / EXCLUSION CRITERIA

Inclusion criteria: children with a diagnosis of Autism Spectrum Disorder, are between the ages of 5 and 12, and like 70% dark chocolate.

Exclusion criteria: Food allergies, Caffeine hypersensitivity, Theobromine hypersensitivity, History of seizures or epilepsy, Developmental age < 24 months, and Diabetics.

6. SUBJECT RECRUITMENT & SCREENING

Based on a power analysis, 35 subjects are needed with an expected attrition rate of 20%. Subjects will include both males and females with an age range between 5 and 12 years. Study will include subjects from any race or ethnicity that meet eligibility criteria. The target study population is school aged children with a diagnosis of Autism Spectrum Disorder. Recruitment will be done via flyers at the Inland Empire Autism Assessment Center of Excellence, which is an interdisciplinary diagnostic center. Upon a formal diagnosis of Autism Spectrum Disorder, the families of those children will be offered a flyer to determine if they are interested in participating in the study. The contact information of the Principal Investigator and graduate student research will be available on the flyer.

7. INFORMED CONSENT PROCESS

The informed consent process will be facilitated by the student researcher with the parents of the child with a diagnosis of Autism Spectrum Disorder. The informed consent process will take place at the child's elementary school and parents will be provided fifteen minutes for decision making and discussion. Due to the subjects' impaired decision making capacity, the parents will provide consent on their behalf. Subjects will be selected based on their diagnosis from the Inland Empire Autism Assessment Center of Excellence. Medical release forms will be obtained via the Inland Empire Autism Assessment Center of Excellence as this center has all baseline and demographic data. HIPPA compliance forms will be included with informed consent process. Privacy of medical and research records will be secured in a locked cabinet in a locked office.

*8.* STUDY DESIGN

a. Background or rationale for this study.

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder characterized by deficits in behavior, communication, and social skills (American Psychological Association, 2013). According to the Center for Disease Control and Prevention (CDC), currently 1 in 68 children are identified as having ASD and it is 4.5 times more common in males than females (CDC, 2016). The etiology is multifactorial as it involves genetics, environment, immunological factors, and oxidative stress factors (Damodaran & Arumugam, 2011; Chauhan & Chahuan, 2006). This is of particular importance to children as the brain is highly susceptible to oxidative stress especially in early childhood development. The reason the brain is susceptible to damage by oxidative stress is because it is the organ that requires the most oxygen for its normal function, it occupies 2% of our body mass yet uses 20% of our metabolic oxygen (Damodaran & Arumugam, 2011; Chauhan & Chahuan, 2006). Thus, any interruption to its oxygen supply greatly impacts its many functions and in the immature nervous system of children protective barriers to oxidative damage are not fully formed hence they are more susceptible. Antioxidants' benefits include improvement of cellular structure, counter free radical damage, neuroprotective, anti-inflammatory, accumulate in hippocampus and cortex, and improve vascular endothelial function (Sokolov, Pavlova, Klosterhalfen, & Enck, 2013; Vauzour, Vafeiadou, Rodriguez-Mateos, Rendeiro,

& Spencer, 2008). More specifically, cacao is a natural and potent antioxidant with benefits linked to being neuroprotective, improves blood pressure, improves lipid peroxidation, improves cognitive response time, enhances gamma frequency, increases cerebral blood flow, anti-inflammatory, and flavanols are able to cross the blood brain barrier (Cooper, Donovan, Waterhouse, & Williamson, 2008; Sokolov, Pavlova, Klosterhalfen, & Enck, 2013; Mastroiacovo et al, 2015; Berk et al, 2017; Vauzour, Vafeiadou, Rodriguez-Mateos, Rendeiro, & Spencer, 2008).

b. Objectives:

    1. To determine if consuming high antioxidant cacao changes the severity of Autistic behaviors;
    2. To determine if parents of children with ASD observe behavioral changes following cacao consumption through behavioral scales;
    3. To determine if teachers of children with ASD observe behavioral changes following cacao consumption through behavioral scales;
    4. To determine if parents' stress level changes following the cacao consumption intervention through stress scale.

c. Procedures involved (Research Interventions)

    1. Informed Consent and HIPPA documents
    2. Subject will also be offered a taste testing of the chocolate bar to be used in the study as liking the 70% dark chocolate is part of inclusion criteria.
    3. After consent is provided, parent will fill out the Youth & Adolescent Food Frequency Questionnaire based on their child's typical dietary habits, an Autism Spectrum Rating Scale, an Aberrant Behavior Checklist, and the Parental Stress Index Questionnaire.
    4. Student researcher will prepare doses (3 squares or 6 grams) of the dark chocolate for each child in one week increments at a time. The dark chocolate will be wrapped in foil to protect against light and kept in a sealed Ziploc bag to prevent against oxidation in order to maintain the cacao's integrity.
    5. Student researcher will meet with each parent/family once per week to collect any questionnaires and to deliver the dark chocolate doses for the subsequent weeks.
    6. Student researcher will meet with teachers at baseline, end of week two, and end of week four to collect all questionnaires.
    7. Subject will consume 3 squares of the dark chocolate every four hours daily for four weeks during waking hours between 8:00 a.m. and 8:00 p.m.
    8. Student researcher will contact parents and teachers weekly via phone/email to follow up on administration of the chocolate.
    9. Parent and primary school teacher will fill out the Autism Spectrum Rating Scale and the Aberrant Behavior Checklist at the end of week two and at the end of week four.

10. Parent will fill out the Parental Stress Index Questionnaire at the end of week four.

d. Concise review of literature that supports the rationale, objectives, and methodology of the proposed study.

Autism Spectrum Disorder involves oxidative stress in the pathophysiology and previous literature has shown that level of oxidative stress markers positively correlated with severity of behavioral symptoms (Damodaran & Arumugam, 2011). Antioxidants, such as cacao, have shown to have great health and neurocognitive benefits on the adult population. In the ASD population, five studies have been conducted looking at an antioxidant intervention on behavior change. Researchers examined use of high antioxidant vitamin C providing 8g/70kg/day for 30 weeks and found a reduction in symptom severity (Dolske, Spollen, McKay, Lancashire, & Tolbert, 1993). It appears this is the first study of its kind to look at antioxidants potential benefit to children with ASD. In a 2013 study, children between the ages of three and a half and 16 were randomly assigned to one of the two groups to receive N-Acetylcysteine (NAC) (1200 mg/day)+risperidone or placebo+risperidone. Results showed a significant improvement in irritability in the NAC group on the Aberrant Behavior Checklist (Ghanizadeh & Moghimi-Sarani, 2013). More recently, in a 2015 study, researchers examined children between four and 12 years of age with a diagnosis of Autism Spectrum Disorder. One group received risperidone plus NAC and the control group received risperidone plus placebo (Nikoo, Radnia, Farokhnia, Mohammadi, & Akhondzadeh, 2015). The dose of NAC ranged from 600 to 900 mg/day. Results showed a significant improvement in both irritability and hyperactivity/noncompliance subscales on the Aberrant Behavior Checklist (Nikoo, Radnia, Farokhnia, Mohammadi, & Akhondzadeh, 2015). The most common adverse effects in the NAC+risperidone group were constipation, increased appetite, fatigue, nervousness, and daytime drowsiness(Nikoo, Radnia, Farokhnia, Mohammadi, & Akhondzadeh, 2015; Ghanizadeh & Moghimi-Sarani, 2013).

Lastly, a 2015 pilot study also assessed NAC in children with ASD between ages three and 12 (Hardan et al, 2015). Subjects were randomized to NAC were initiated at 900mg/daily for four weeks, then 900mg/twice daily for four weeks and 900mg/three times daily for four weeks (Hardan et al, 2015). Follow-up data revealed that the NAC group showed significant improvements on the Aberrant Behavior Checklist irritability subscale compared to the control group (Hardan et al, 2015). Also, stereotypy behaviors improved on the repetitive behavior scale and social cognition improved on one of the subtests on the social responsiveness scale (Hardan et al, 2015). These five studies are key in showing some of the reproducible evidence of potential therapeutic effects of antioxidant interventions in children with ASD. To date no study has analyzed the potential effects of high antioxidant cacao as an intervention for behavior change in the ASD population.

9. DATA COLLECTION

Data collection procedures include psychometric assessments of common behaviors found in children with Autism Spectrum Disorder. They will be completed by child's parent and primary teacher. The Youth & Adolescent Food Frequency Questionnaire will be administered to parent at baseline to determine their child's typical dietary habits. The Aberrant Behavior Checklist and Autism Spectrum Rating Scale will be administered to parent and primary teacher at baseline, end of week two, and end of week four to measure behavior change. The Parental Stress Index will be administered to the parent at baseline and end of week four to determine if caregiver stress changed over the course of the intervention. All assessment tools are self-report measures using ordinal data. Demographic data will also be obtained from the Inland Empire Autism Assessment Center of Excellence. Further, the purpose of collecting medical chart information is to gather demographic data on whether the child has received any therapeutic interventions and if so, type of therapy and length of time. Age, medications, years in school, developmental factors and milestones, medical history, and social history will also be collected. These demographic data would be used in data analysis to determine the influence or associations of these variables on study outcomes.

See attached appendices of: Aberrant Behavior Checklist, Autism Spectrum Rating Scale (short form), Parental Stress Index (short form), Youth & Adolescent Food Frequency Questionnaire, and Demographic Data. Short forms are provided to give the nature of the questions and the long forms are in the process of being ordered.

10. LABELING & STORAGE OF DATA & SPECIMENS

Informed consents and HIPPA documents will be stored in a locked cabinet in a locked office. Any electronic research records of behavioral scales, stress scales, and any other research information will be stored on a secured and password protected computer. All hard copies of research records of behavioral scales, stress scales, and any other research information will be kept in a locked cabinet in a locked office. Data will be secured in a locked cabinet for a period of three years.

11. DATA ANALYSIS

Research analysis plan will include non-parametric statistics. For the pre-test and post-test analysis of the Parental Stress Index scale Friedman's test will be used. For all other repeated measures such as the Aberrant Behavior Checklist and the Autism Spectrum Rating Scale the Wilcoxon Signed Rank Test will be used.

12. RISK AND INJURY

Risks may include a breach of confidentiality and parent or teacher may feel uncomfortable answering questions.

13. BENEFIT(S)

Although you may not personally benefit from this study, your participation may help clinicians and teachers when working with children with a diagnosis of Autism

Spectrum Disorder. Results of the study can provide insights to practitioners and teachers while working with children with Autism Spectrum Disorder.

14. COMPENSATION

Families who complete the study in full will receive a $50 gift card at the end of the study for completing it in full.

15. CONFIDENTIALITY

All records and research materials that identify you will be held confidential. Any published document resulting from this study will not disclose your identity without your permission. Information identifying you will only be available to the study personnel. All data will be secured in a locked cabinet in a locked office.

16. LITERATURE REVIEW

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder that is characterized by deficits in social skills, communication skills, functional impairments across multiple settings, repetitive and restrictive behaviors, as well as hyper or hypo responsiveness to sensory stimuli in the environment (American Psychiatric Association, 2013). According to the Center for Disease Control and Prevention, 1 in 68 children were labeled as having ASD (CDC, 2016). Further, it is four and a half times more common in males than females (CDC, 2016). Although the etiology of ASD remains elusive, there are genetic, environmental, immunological, and oxidative stress influences that are related to the pathogenesis of the disorder (Damodaran & Arumugam, 2011).

Oxidative stress is the excess accumulation of free radicals in the body. Oxidative stress has been linked to a variety of diseases including cancer, cardiovascular disease, neurological diseases, pulmonary disease, rheumatoid arthritis, nephropathy, as well as ocular disease (Pham-Huy & Pham-Huy, 2008). Oxidative stress has also been under study as part of the pathophysiology of ASD and is linked to neuropsychiatric disorders such as schizophrenia, major depressive disorder, anxiety, panic disorder, and obsessive compulsive disorders (Damodaran & Arumugam, 2011). The human body offers mechanisms to counter the effects of free radicals, that lead to oxidative stress, by producing antioxidants. Antioxidants can be

produced naturally by the body or can be ingested through dietary nutrients. Examples of dietary antioxidants include Vitamin E, Vitamin C, Beta-carotene, Lycopene, Selenium, Omega-3 fatty acids, Omega-6 fatty acids, and Flavonoids (Pham-Huy & Pham-Huy, 2008). Flavanoids have a potent antioxidant content and are derived from foods such as cocoa, green tea, grapes, apples, ginkgo biloba, berries, among other foods (Pham-Huy & Pham-Huy, 2008).

Research suggests that children with ASD tend to have greater markers of oxidative stress compared to their age matched controls or their non-Autistic siblings (Ming et al, 2005). Oxidative stress can be due to mitochondrial impairment, environmental factors, metabolic issues, and genetic predisposition (Ming et al, 2005). In turn, oxidative stress can lead to a cascade of events such as inflammation, damage to cell membranes, autoimmunity, methylation damage, cell death, and neurological impairments (Goldani, Downs, Widjaja, Lawton, & Hendren, 2014). The brain is an organ highly susceptible to oxidative stress which is of particular importance in early child development (Chauhan & Chauhan, 2006). Oxidative stress can be identified through markers of antioxidant enzymes, lipid peroxidation, and protein oxidation which are all higher in children with autism compared to their healthy peers (Damodaran & Arumugam, 201; Ming et al, 2005).

Researchers observed a significant increase in oxidative stress markers in children with ASD compared to matched controls and the level of antioxidants excreted in urine samples were significantly lower in children with ASD compared to matched controls (Damodaran & Arumugam, 2011). Further, the level of severity of autistic behaviors were positively correlated with oxidative stress markers and antioxidant levels revealed a negative correlation with autistic behaviors (Damodaran & Arumugam, 2011). Ming et al (2005) measured urinary excretion of 8-iisoprostane and 8-hydroxy-2-deoxyguanosine in children with ASD and matched controls and found children with ASD had increased levels of lipid peroxidation. Elevated blood samples of glutathione, glutathione peroxidase, methionine, oxidized glutathione, and cysteine have also

been found in children with ASD (Goldani, Downs, Widjaja, Lawton, & Hendren, 2014). Further, the severity of ASD was correlated with a lower level of antioxidants (Goldani, Downs, Widjaja, Lawton, & Hendren, 2014).

Oxidative stress biomarkers are particularly noteworthy as they have the potential to be modulated by means of non-pharmacological interventions, such as nutritional interventions. Cocoa is a food rich in flavonoids and their sub group flavanols. These are both associated with improved cognitive processing, alertness, and processing speed (Crichton, Elias, & Alkerwi, 2016). Further benefits of cocoa include increased cerebral blood flow, improved cardiovascular activity, improved insulin resistance, improved blood pressure, and improved endothelial function (Crichton, Elias, & Alkerwi, 2016).

Current behavioral interventions that are utilized for children with ASD include behavioral therapists, speech and language pathologists, occupational therapists, and pharmacological interventions. Behavioral therapists work with ASD children on language skills, daily living skills, and social functioning, however, it should be noted that this type of therapy uses a Skinner approach with rote discrete trials in a one on one setting and is typically recommended at 40 hours per week for years on end (Peters-Scheffer, Didden, Korzilius, & Sturmey, 2011; Virués-Ortega, 2010). Speech and Language Pathologists also work with children with ASD to target social communication skills, request making, speech production, and reciprocal interaction (American Speech-Language-Hearing Association, 2006). Occupational therapists target sensory processing skills as well as motor skills (Case-Smith & Arbesman, 2008). In terms of pharmacology, there is no approximately 1/3 of the ASD population take Selective Serotonin Reuptake Inhibitor drugs because their behavioral characteristics are similar to other serotonin related disorders. There are only two Food & Drug Administration (FDA) approved drugs for ASD are Abilify and Risperidone (LeClerc & Easley, 2015). Risperidone is a second-generation antipsychotic that has helped children with ASD improve number of tantrums,

aggressive and self-injurious behaviors and Abilify is a psychotropic drug that was designed to treat irritability in children with ASD (LeClerc & Easley, 2015). However, as with any drug they come with adverse effects including increased appetite, weight gain, dizziness, drooling, drowsiness, fatigue, vomiting, nasopharyngitis, fever, insomnia, and upper respiratory infections (LeClerc & Easley, 2015).

Many antioxidant rich foods and vitamins have been used in the treatment of disease (Li, Chen, Zhang, Wu, Wu, & Liu, 2014). For example, vitamin C and vitamin E are both rich in antioxidants and have been used for cardiovascular disease and cancer (Li, Chen, Zhang, Wu, Wu, & Liu, 2014). Carotenoids have also been used in diseases that are neurodegenerative, inflammatory, or cardiovascular in nature (Li, Chen, Zhang, Wu, Wu, & Liu, 2014). Polyphenols, such as those found in cocoa, are known to have the ability to remove free radicals from the body and can offer protective properties in disease such as arteriosclerosis, heart disease, and alcoholic fatty liver (Li, Chen, Zhang, Wu, Wu, & Liu, 2014).

To date, research has not examined the potential association between antioxidant rich foods, such as cocoa, and ASD behaviors. However, it is expected, based on the literature reviewed, that oxidative stress markers, such as free radicals, are elevated in children with ASD and thus could be reduced by high antioxidant cacao consumption. Thus, the purpose of the study is to provide information on the relationship between eating high antioxidant cocoa relative to behavior in children with ASD.

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