IMPACT OF BIOACTIVE COMPOUNDS ON NEUROCOGNITIVE DEVELOPMENT AND METABOLISM

by

XI FANG

(Under the Direction of HEA JIN PARK)

ABSTRACT

Bioactive compounds are constituents in foods or dietary supplements, other than those needed to meet basic human nutritional needs and, are responsible for changes in the health status. Commonly studied bioactive compounds include polyphenols, alkaloids, carotenoids, terpenoids, and omega-3 and polyunsaturated fatty acid (PUFA). They exhibit anti-inflammatory, antioxidative, antidiabetic, anticancer, antimicrobial, and other health benefits that positively influence the biological functions such as the immune, endocrine, and nervous systems. Bioactive compounds such as phenolic compounds, carotenoids, and ω3 polyunsaturated fatty acids are reported to support brain development and neurocognitive function. They also play a pivotal role in regulating metabolic functions such as insulin sensitivity, glycemic control, inflammation, and oxidative stress. The objective of this dissertation is to examine the roles of bioactive compounds in neurocognitive development and metabolic functions. In manuscript #1, the effects of perinatal docosahexaenoic acid (DHA) supplementation on the neurocognitive function were examined by behavioral tests, Diffusion Tensor Imaging (DTI), and functional Magnetic Resonance Imaging (fMRI) in a sow/piglet dyad model. Perinatal DHA intake enhanced exploratory behaviors and cognitive function, increased hippocampal fiber length, and

altered brain functional connectivity. In manuscript #2, impacts of green tea extract (GTE) and regulator of G-protein signaling 10 (RGS10), a negative immune modulator, on high-fat-diet (HFD)-induced metabolic dysfunction were examined in a mice model. The absence of RGS10 protein exacerbated HFD-induced weight gain, glucose metabolism, and inflammation, which was ameliorated by GTE. Since the efficacy of bioactive compounds may be limited by their low bioavailability, manuscript #3 examined the potential synergistic effects of single-dose GTE plus lemon juice (LJ) on circulating green tea catechin and antioxidative function in a pig model. The co-intake of LJ with GTE increased plasma levels of (−)-epigallocatechin3-gallate (EGCG) and (−)-epigallocatechin (EGC), whereas it did not affect oxidative stress and lipid metabolism. Findings from these studies provide valuable insights into the therapeutic roles of bioactive compounds in neural and metabolic regulations that may benefit brain development and obesity-related metabolic syndromes.

INDEX WORDS: DHA, GREEN TEA, COGNITIVE FUNCTION, BRAIN DEVELOPMENT, MAGNETIC RESONANCE IMAGING, FUNCTIONAL MAGNETIC RESONANCE IMAGING, DIFFUSION TENSOR IMAGING, GLUCOSE TOLERANCE, INSULIN RESISTANCE, INFLAMMATION, BIOAVAILABILITY, OXIDATIVE STRESS, LIPID PROFILE

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XI FANG

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XI FANG

Major Professor:	HEA JIN PARK
Committee:	MICHAEL J. AZAIN
	JAE-KYUNG LEE
	ROBERT PAZDRO
	SRUJANA RAYALAM

Electronic Version Approved:

Ron Walcott
Dean of the Graduate School
The University of Georgia
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CHAPTER 1

INTRODUCTION

Bioactive food components are constituents in foods or dietary supplements, other than those needed to meet basic human nutritional needs, that are responsible for changes in health status [1]. Bioactive compounds that are heavily investigated include polyphenols alkaloids, carotenoids, terpenoids, and omega-3 and polyunsaturated fatty acids (PUFA). Polyphenols include phenolic acids such as caffeic acid, flavonoids such as catechins, stilbenes such as resvertrol, and lignans such as secoisolariciresinol [2-5]. Bioactive compounds are suggested to influence various biological systems and exhibit anti-inflammatory, antioxidative, antidiabetic, anticancer, antimicrobial functions [6].

Prenatal and early postnatal time represent critical periods of brain development sensitive to nutritional status [7]. Docosahexaenoic acid (DHA) is an omega-3 polyunsaturated fatty acid (ω3 PUFA) commonly recommended during pregnancy and is widely added to the infant formula due to its suggested beneficial role in visual function and cognitive development [8,9]. DHA accounts for more than 40% of the total n3 PUFA in the neuronal tissue [10] and accumulates most rapidly during the third trimester of the gestation period, into the postnatal life [11,12], coinciding with the rapid myelination, dendritic outgrowth, and synaptogenesis [13], suggesting the importance of DHA during the critical window of neurodevelopment. In humans, compelling evidence from observational studies supports the positive association between maternal fish intake and infant DHA status in neurocognitive development [14-16]. However, intervention trials supplementing DHA during gestation and/or lactation yielded mixed and conflicting findings in healthy term infants [17-21] likely

due to the heterogeneity of the study designs. In addition, it remains unexplored whether perinatal DHA supplementation impact brain structural and functional organization using advanced neuroimaging techniques such as magnetic resonance imaging (MRI). This discrepancy urges further elucidation of the roles of DHA on neurocognitive function and brain functional organization in highly translatable animal models.

Diet-induced obesity and its related metabolic syndromes, often accompanied by elevated systemic inflammation and impaired insulin sensitivity, constitute severe threats to human health as significant risk factors for chronic diseases such as cardiovascular disease and type 2 diabetes mellitus [22,23]. Green tea (*Camellia sinensis*) is a popular beverage consumed worldwide for its long history and numerous health benefits. Green tea has been known for the protective activity against obesity [24,25], cardiovascular disorders [26,27], diabetes [28,29], and various types of cancers [30,31] through exerting its antioxidant and anti-inflammatory effects. The major polyphenolic compounds in green tea are (−)-epigallocatechin gallate (EGCG), (−)-epicatechin-3-gallate (ECG), (−)-epigallocatechin (EGC), and (−)-epicatechin (EC), among which EGCG is the most abundant, accounting for more than 50% of catechin [32]. Green tea and its catechins exhibit antioxidative and anti-inflammatory properties, making them potentially potent therapeutics for diseases where these processes are critical negative elements [33-37]. Green tea attenuates high-fat diet (HFD)-induced obesity and metabolic dysfunction characterized by increased oxidative stress, blood pressure, glucose intolerance, and pro-inflammatory signaling molecules such as TNF [38,39]. In addition, the Regulator of G-protein signaling protein 10 (RGS10), a negative inflammatory mediator in the brain and periphery [40,41] is implicated to be a therapeutic target of green tea for metabolic disorders modulating both inflammation and metabolic homeostasis.

Experimental animal models have contributed heavily to human nutrition research to understand the impacts of food on human health and disease. Mice are the most commonly used model and the mouse model of diet-induced obesity remain one of the most useful tools to unravel the interaction between diet and the progression of obesity-related metabolic abnormalities in human [42,43]. Pigs are emerging as a useful model for both nutritional and neurodevelopmental research due to a myriad of similarities to humans in physiology, anatomy, pathology, and eating behavior [44-47]. Pigs have a gyrencephalic brain [48,49], a key architectural difference that has a direct correlation with brain connectivity and complexity [50-53]. The human and swine brain is composed of >60% white matter [54,55] and anatomical similarities in different brain regions, such as the HC [56], subcortical and diencephalic nuclei [57], and cortical regions [58,59] between the pig and human brain have been illustrated. Furthermore, the pig brain exhibits a perinatal growth spurt, much like human infants during which the brain weight grows most rapidly from the last trimester of gestation to lactation [60,61]. The use of animal models such as rodents and pigs provides valuable tools to investigate the interactions between nutrition and human health and diseases.

The literature review (Chapter 2) provides an overview of the current body of evidence relating to the following topics: 1) neurodevelopment and perinatal nutrition, 2) DHA and neurocognitive development, 3) DHA and gut microbiome 4) DHA and gut-brain crosstalk, 5) obesity and metabolism, 6) green tea and antiobesity effects, 7) Bioavailability of bioactive compounbds. Chapter 3 presents a study examining whether maternal supplementation of DHA during late gestation and lactation imparts advantages to cognitive development, fiber bundle maturation, anatomical and functional organization, and the monoamine neurotransmitter status of the developing brain in healthy offspring using a sow/piglet dyad model. Chapter 4 is a study conducted in high-fat diet induced obese mice that examined the roles of the regulator of G-protein

signaling 10 (RGS10) and green tea extract on insulin sensitivity, and inflammation. Although various bioactive compounds have been suggested to ameliorate diet-induced obesity, inflammation, and oxidative stress, their efficacy is limited due to the low bioavailability after ingestion that may be enhanced by co-intake with other bioactive compounds to maximize the health benefits. Chapter 5 presents a study investigating the potential synergistic effects of green tea and lemon juice on circulating catechins and antioxidative capacity combined with low-fat or HFD in a pig model.

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CHAPTER 2

REVIEW OF THE LITERATURE

Neurodevelopment and Perinatal Nutrition

Human infant brains undergo rapid perinatal neural growth spurt from late gestation to the first few years of life [1,2]. Extending from embryonic, fetal, postnatal stages, to even later in life, the brain undergoes complex processes including neurogenesis, neuronal migration, gliogenesis, myelination, synaptogenesis, apoptosis, and synaptic pruning and form critical neural circuits that serve fundamental functions [2]. During gastrulation, epiblast layer cells differentiate into neural stem cells (NSCs, also called neural progenitor cells (NPCs)), which is capable of producing all neuronal cells in the central nervous system (CNS) [3]. Asymmetrical division of NPCs produces NPCs and neuroblasts [3], which then migrate from the ventricular zone to the developing neocortex, differentiate into neurons, forming an orderly laminar organization, and integrate into the neural network [3,4] extending from E42 to around mid-gestation stage for most brain areas [3]. While postnatal neurogenesis is very limited, glial progenitor cells can still migrate and differentiate into astrocytes and oligodendrocytes as they translocate from the subventricular zone (SVZ) outwards into the striatum, hippocampus, and cortex region, possibly through later in postnatal life [5,6]. Myelin, originated from oligodendrocytes in CNS and Schwann cells in PNS, is a modified plasma membrane wrapped around the axon of neurons facilitating the signal conduction [7]. Myelination occurs as early as 29th week of gestation, grows most rapidly from last trimester until early infancy where most major tracts are sufficiently myelinated by early childhood while some persists later in life [2,8,9].

Synaptogenesis occurs around 20th week of gestation period, increases rapidly through late gestation and early postnatal period where the maximum synaptic density is reached in the early infancy [9].

To support the rapid development, the neonatal brain is of high energy demand and consumes up to 60% of the body's calorie [10]. Therefore, the rapidly growing brain is vulnerable to developmental deficits if adequate nutrition is not provided that can cause long-term functional deficits [10,11]. Critical nutrients widely studied that influence brain development include macronutrients such as long-chain polyunsaturated fatty acids (LCPUFAs) and protein, and micronutrients such as iron, iodine, zinc, and vitamin B12 [11]. Perinatal protein deficiencies resulted in altered neurobehaviors, cognitive impairment, decreased antioxidant capacity, and altered neurotransmitters in the developing brain [12]. Antioxidants such as vitamin E concentrations in the mothers are related to fetal growth, congenital heart defects, and other birth defects [13]. Omega-3 PUFA deficiency during the perinatal stage is associated with altered microglia-mediated neuroinflammation [14] and memory impairment [15]. Minerals like iron are shown to affect motor development [16], social-emotional behaviors [17], and auditory functions in infants [18] while iodine deficiency is related to thyroid dysfunction and mental retardation [19].

DHA and Neurocognitive Development

Maternal DHA Supplementation on Infant Brain and Cognitive Development

DHA accumulated most rapidly during the perinatal stage, and breast milk is an ideal source of DHA for the infant, the concentration of which is reflective of dietary intake [20]. It is estimated that the DHA content in human breast milk is approximately 0.32% [21], and supplementing the diet of lactating women with DHA significantly improved its level in the breastmilk [22]. The first study investigating infant neural function after maternal DHA

supplementation conducted by Gibson et al. [23] found a dose-dependent rise in infant plasma phospholipids and erythrocyte phospholipids DHA with maternal DHA intake (algal oil after delivery for 12 weeks) but no improvement in visual acuity in infants at 12 and 16 weeks. Later studies with similar treatment period in Danish mothers (900 mg/d DHA) conducted by Lauritzen et al. and American mothers (200 mg/d DHA) by Jensen et al. also failed to detect an improvement in visual function, but the Danish study found it to be associated with infant erythrocyte DHA at 4 months while Jensen et al. found maternal DHA supplementation improved performance in Bayley Psychomotor developmental index (PDI) at 30 months and better sustained attention at 5 years of age [24-28].

Investigations into prenatal DHA supplementation on visual and cognitive function yielded mixed findings that may be related to the population of interest. Supplementing pregnant women fish oil containing DHA (0.2-2.2 g/d) during the third trimester of gestation for 3.75-5 months did not influence any of the cognitive-developmental or behavioral measures in a study conducted in Bangladesh [29] but was found to benefit visual acuity at 2 and 4 months [30], intention at 9 months [31], attention at 1 and 2 years [32,33], and language development at 14 and 18 months of age [34,35] in studies based in North America, although different control diets were used that may partly contribute to the discrepancy. In an Australia study, fish oil intake (2.2 g DHA and 1.1 g EPA) during the second half of pregnancy improved eye and hand coordination at 2.5 years [36] but did not influence language, cognition, or fine-motor control at 12 years of age [37]. However, the DHA to Optimize Mother Infant Outcome (DOMInO) trial conducted in South Australia (800 mg/d DHA during the last half of gestation) and a study in Mexican population (400 mg/d algal DHA from mid-gestation) observed no difference in cognitive and language scores [38],

attention and working memory [39], visual acuity [40], or psychomotor indices between the treated and untreated groups [41].

Maternal supplementation of DHA (1183 mg DHA, 803 mg EPA) during both gestation and lactation periods resulted in a similar performance in visual acuity and Fagan novelty test in infants within the first year; however, umbilical plasma DHA was indicative of a more mature electroencephalogram (EEG) score at birth [42] in the Norwegian population. In the 4-year follow-up, supplemented infants showed better mental processing composite of the Kaufman Assessment Battery for Children (K-ABC), which was associated with plasma DHA at 4 weeks [43]. DHA dosage in this study (1183 mg/d) is much higher than the later studies (220-320 mg/d), which did not observe a change in any neurological changes at 2.5-18 months after birth following 7-9 months supplementation [44,45]. Apart from the apparent dosage difference, it is also likely that the effects of DHA become more prominent later in life, and the measurements may not be sensitive enough to detect the change earlier.

Postnatal DHA Supplementation on Healthy Term Infant Brain and Cognitive Development

Although term infants undergo intrauterine DHA accretion during the last trimester, brain and circulatory DHA status after birth are largely dependent on postnatal DHA intake from breastmilk or infant formula [46,47]. Early investigations found DHA (0.35%) and DHA+ arachidonic acid (AA) (0.36% DHA+ 0.72% AA) supplementation for 4-13 months lead to improved sweep visual evoked potential (VEP) visual acuity [48-51] and cognitive and motor function at 12 months of age [52], in accordance with other studies [53,54]. However, some studies found either no effect or a transient improvement in visual function at 2 months [55], which was not seen at later ages around 4-9 months [47,55-57]. Noteworthy, studies may vary in their methods used for VEP estimates such as transient or steady-state VEP and stimulation with checkerboard

or square wave gratings [47,55-57], sources of DHA such as fish oil or egg yolk [55,58], other LCPUFA content in the regiments such as eicosapentaenoic acid (EPA) and arachidonic acid (AA) [48,49], intervention initiation time of whether starts immediately after birth or a few months after birth [53,54], or population characteristics such as ethnicity and geographic location [47,55,56], that may contribute to the contradictory findings.

Function beyond the visual system, postnatal DHA intake may influence different mental and cognitive measures in healthy infants. Early DHA supplementation in term infants was found to exert positive effects on problem-solving ability at 9-10 months [59,60], mental development at 18 months [52,61], and sustained attention at 4-9 months of age [62], while others found marginal or no effect on communicative development [63], visual-motor function [64], or psychomotor development [52,57]. The looking times during infant visual habituation, indicative of information processing speed [65], and problem-solving skills were suggested to be related to childhood IQ [66]. Indeed, infants supplemented with DHA and AA for 4 months was found to have lower total looking times at 3 months [67] and better problem-solving skills at 9-10 months and led to similar verbal IQ to breastfed infants at 4 years [68]. In one study, Auestad et al. did not observe any group difference in infant IQ at 3 years of age [69], mental and motor development at 12 months [70], and the treatment group had lower vocabulary production and vocabulary comprehension at 14 months [70]; this discrepancy may be attributed to a lower dosage (0.12% DHA) compared to other studies. A large multicenter randomized control trial (RCT) conducted between 1992-2013 in Europe found DHA and AA supplementation (0.15-0.25% DHA, 0.3-0.4% AA) during the first 4 years resulted in a better performance in Brunet-Lézine psychomotor development test at 4 which disappeared at 24 months [71,72], improved problem-solving skills at 9-10 months [59,60], and faster information processing skill at 6 years of age [73]. The DHA Intake And Measurement Of

Neural Development (DIAMOND) study supplemented infants with 0%, 0.32%, 0.64%, or 0.96% DHA combined with 0.64% of AA for 1 year and were followed up for 9 years. Infants fed the supplemented formula had higher sweep VEP visual acuity at 12 months [74], increased mental development index at 18 months [61], better inhibitory function from 3-5 years [75], and better verbal and full-scale IQ at 6 years [75] compared to the unsupplemented infants. Infants from the two intermediate dosages had better sustained attention from 4-9 months [62], executive function from 3-5 years [75], and verbal intelligence quotient at 5 years [75] compared to the control infants. At later follow-ups, electrophysiological and MRI measurements revealed early-life DHA supplementation had long-term effects on brain electrophysiology at 5.5 years [76] and led to greater activation in the anterior cingulate cortex (ACC) and parietal regions during inhibition tasks, greater connectivity between prefrontal and parietal regions, and larger white matter volume in ACC at 9 years [77], supporting that the attention and inhibition systems are sensitive to early life DHA status.

In summary, evidence of improved visual function, cognitive and psychomotor development following intrauterine and postnatal DHA intake in healthy infants were reported, although mixed findings were also commonly seen, likely due to the heterogeneity of the study design, including feeding duration, sources and dosage of DHA supplementation, levels of other fatty acids in the supplementation, age when tested, neurocognitive domains of interest, measurements for early cognitive development, the population of interest and baseline characteristics of each trial. Generally, positive effects were more commonly seen in trials supplementing infants directly than through the maternal intake [78,79], which may be partly on account of different control groups they are compared to. It is important to note that trials supplementing infants usually compare infants fed formula with or without DHA while the

maternal supplementation trials usually compare those infants with healthy infants who exclusively received breastmilk, the gold standard for optimal nutrition, and contains some amount of DHA and other PUFAs [27,74].

DHA and Gut Microbiome

Prenatal and early postnatal period is characterized by a critical and sensitive time of infant brain development, coinciding with rapid development and maturation of the microbial community [80]. The gut microbiome has increasingly become a focus of preclinical and clinical studies of neurocognitive and emotional development. A recent human study showed that infants who had a higher proportion of their microbial population comprised of the genus Bacteroides displayed better gross motor skills, perceptual abilities, and language development [81]. These results highlight an association between cognitive development and the gut microbiome of infants that may underlie the beneficial effects of DHA on infant brain development.

Early in life, premature infants who received enteral supplementation with fish oil and safflower oil had increased alpha-diversity and lowered Clostridium, Streptococcus, and bacteria within the Enterobacteriaceae family compared to the infants who received standard care [82]. Similarly, human breast milk DHA level was positively correlated with Enterobacteriaceae and Bacteroides [83], supporting the dietary ω3 LCPUFA intake influences the gut microbiome ecology during development.

DHA is commonly reported to influence the short-chain fatty acid (SCFA)-producing microbiome. Specifically, Lachnospiraceae and Ruminococcacae families hydrolyze complex polysaccharides such as fiber and resistant starch to generate short-chain fatty acid (SCFA), including butyrate, acetate, and propionate that are utilized for energy and exert beneficial effects on health [84,85]. Serum DHA level was positively correlated with microbiome diversity,

Lachnospiraceae, Ruminococcacae, and Bacteroidetes abundances [86], and DHA supplementation increased SCFA-producing microbiota including Roseburia, Lanchnospira, Coprococcus, Roseburia, Anaerostipes, and Oscillospira [87,88].

While the effects of DHA on the gut microbiome is by no means a simple direction and is largely dependent on the individual and their baseline health status, some common changes are highlighted. Common phylum including Bacteroidetes [83,88], Firmicutes[88-90], Actinobacteria [82,88,91], and Proteobacteria [82] are all indicated to be associated with DHA status. At the family level, Lachnospiraceae [83,86,89,92] and Ruminococcaceae [86,92] from Firmicutes Phyla are the ones mostly reported to be influenced by DHA intake in human. Positive associations between DHA status and genus Lachnospira [83,86,87], Roseburia [86-88] from Lachnospiraceae family, Ruminococcus [86,88] and Oscillospira [86,87,92] from Ruminococcaceae family, and Bifidobacterium [87,91,92] and a negative effect in Faecalibacterium [87-89] are often observed in human. Importantly, DHA was shown to influence main SCFA producers such as Roseburia, Faecalibacterium, Ruminococcus, Blautia, Coprococcus, and Clostridium which may be an important regulator of the interplay between dietary DHA intake, gut microbiota, and host immune function.

DHA and Gut-brain Crosstalk

DHA has been shown to influence brain development and cognitive function and influence gut microbiome composition, which is in close functional crosstalk with the central nervous system and influences brain function [93]. It is plausible that the neuroprotective effects of DHA is elicited partly through the commensal bacteria resides in the gastrointestinal (GI) tract. Several pathways have been suggested relating the neuro-supportive effects of DHA and gut microbiome, including the HPA axis, neuroinflammation, and neuroactive metabolites.

DHA intake has been linked to an improved neurocognitive function through the regulation of the hypothalamic-pituitary-adrenal (HPA) response to stress [94]. DHA status during development is effective in mediating HPA activity, reducing depressive- and anxiety-like behaviors, and influence cognitive function such as learning and memory [94-97]. While ω3 PUFA deficiency induces chronic stress and emotional impairment through HPA axis hyperactivity characterized by elevated circulating corticosterone levels and disrupted glucocorticoid receptors (GR) signaling [95,98], ω3 PUFA supplementation normalizes the dysregulated HPA activity, attenuates the chronic stress-induced anxiety-related behaviors, and prevents the stress-mediated learning and memory impairment [98,99]. At the current stage, limited studies investigate the interaction between dietary DHA supplementation and gut microbiome composition in relation to HPA stress response. Nonetheless, evidences suggest DHA supplementation restored maternal separation-induced microbial dysbiosis, normalized inflammation-related bacteria, increased beneficial bacteria, reduced corticosterone response to acute stress, and benefit cognitive memory that persisted later in life [100-102]. These studies provide initial evidence suggesting that DHA supplementation sufficiently modulates gut microbiome dysbiosis in response to stress, regulates HPA-axis activation, and ameliorate stress-induced behavioral and cognitive deficit.

DHA may affect brain function through the microbe-immunity nexus by influencing the inflammation-regulatory gut microbiome. Serum ω3 PUFA was found positively correlated with gut microbiome diversity and negatively with bacteria which are negative modulators of intestinal inflammation such as Prevotella, Coprococcus, Clostridium, Roseburia, Ruminococcus, and Lachnospiraceae [86,103]. Omega 3 PUFA is reported to increase anti-inflammatory bacteria such as *Bifidobacterium*, *Lactobacillus,* and *Akkermansia muciniphila* and decrease inflammation-inducing bacteria such as *Enterobacteriaceae*, segmented filamentous

bacteria (SFB), *Bilophila*, and Clostridia spp that downregulates inflammatory responses primarily through toll-like receptor (TLR) signaling [104,105]. Collectively, the well-documented anti-inflammatory properties of omega 3 fatty acids may be partly attributable to its interaction with the host gut microbiota, which functions as an important immune modulator in the inflammatory response.

The beneficial effects of DHA on cognitive function has been largely associated with its regulatory roles in neuroactive mediators, including neurotransmitters that enable neuronal communication through chemical synaptic transmission [106-108], neurotrophic factors that support neuronal survival and differentiation and synaptic plasticity essential for memory [109,110], as well as microbial metabolites SCFAs that influence microglia homeostasis and neuronal function by modulating neurotransmitters and neurotrophic factors [85]. As elaborated in the previous session, dietary DHA is a pivotal mediator of SCFA-producing bacteria in the human gut, including families *Lachnospiraceae* and *Ruminococcacae*, genera *Roseburia, Lanchnospira, Clostridium, Anaerostipes, Subdoligranulum, Blautia, Ruminococcus, Faecalibacterium*, and *Coprococcus* [83,86,87,92]. Through anaerobic fermentation of dietary fiber and other indigestible polysaccharides, gut microbiota produces SCFAs which travel across the blood-brain-barrier (BBB) and influence BBB integrity, microglia homeostasis, neurotransmission, neurogenesis, and neuroplasticity that contributes to the improved brain function [111]. For instances, sodium butyrate and acetate have been shown to induce microglial process elongation, regulate microglia-mediated inflammatory signaling, and modify endotoxin-induced depression-like behaviors [112-116]. In addition, SCFAs regulate neuronal communication by mediating the synthesis and signaling of neurotransmitters such as serotonin (5-HT) [117], dopamine (DA) [118], norepinephrine (NE) [119], as well as neuroplasticity by modifying the transcription and expression of neurotrophic factors such as

nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) [120,121]. These evidences suggest neuroactive metabolites such as neurotransmitters, neurotrophins, and SCFAs as critical ways through which DHA regulates brain function.

Obesity and Metabolism

Obesity is an increasing concern with a prevalence of 42.4 % among U.S adults, according to the National Health and Nutrition Examination Survey in 2017-2018 [122]. Often accompanied by elevated systemic inflammation and impaired insulin sensitivity, obesity and its related metabolic syndromes constitute severe threats to human health as major risk factors for chronic diseases [123,124]. Diet remains the most modifiable factor for obesity, and high-fat diet (HFD) feeding is related to obesity and its related metabolic dysfunction [125-127]. HFD-fed animals exhibit higher circulating fasting glucose and insulin, impaired glucose tolerance, and insulin resistance [126]. HFD is also related to altered fatty acid metabolism. Excessive fat intake increases chylomicrons production from the intestine and free fatty acids (FFA) released into the blood, which is incorporated into very-low-density lipoproteins (VLDL) in hepatocytes and subsequently produce low-density lipoproteins (LDL) [128]. The excess production of LDL into the blood and a lack of LDL receptors in hepatocytes lead to LDL being oxidized to oxidized LDL (oxLDL), engulfed by macrophages, which subsequently turns into foam cells and induce cytokines release and inflammation [129]. Increased adiposity following HFD increases the production of reactive oxygen species (ROS), cytokines, and chemokines such as interleukin (IL) 6, IL1β, IL6, IL10, tumor necrosis factor (TNF) α, and monocyte chemoattractant protein-1 (MCP-1/CCL2) [127,130,131]. In summary, HFD-induced overweight and obesity is associated with increased adiposity, altered lipid metabolism, impaired insulin sensitivity, oxidative stress, and elevated inflammation.

Green Tea and Antiobesity Effects

Green tea catechins are polyphenols derived from unfermented leaves of plant *Camellia sinensis* with Epigallocatechin 3-gallate (EGCG), Epicatechin 3-gallate (ECG), and Epigallocatechin (EGC) being the most abundant catechin in green tea. [132] There is a vast amount of studies reporting the beneficial effects of green tea on body weight and body composition following approximately 12 weeks of intake at a dosage of 600-900 mg/day, which is equivalent to 3-4 cups of green tea, in various population [132,133]. The antiobesogenic effects of green tea is speculated to be primarily due to its action on promoting energy expenditure and fat oxidation while downregulating adipogenesis, nutrient absorption, and appetite [134-136]. In addition, green tea exerts favorable effects on obesity-related metabolic conditions such as insulin resistance, hyperlipidemia, inflammation, and oxidative stress. Green tea was reported to improve glycemic control and insulin sensitivity and reduces fasting glucose and hemoglobin A1c concentration [137]. In subjects with type 2 diabetes mellitus and lipid abnormalities, 1500 mg green tea every day for 4 months significantly decreased triglycerides and insulin resistance index and increased high-density lipoprotein cholesterol and glucagon-like peptide 1 [138]. HFD feeding induced hyperlipidemia, hyperglycemia, and insulin resistance, and green tea was demonstrated to effectively improve insulin sensitivity, reduce blood pressure, and decrease blood lipid level in a dose-dependent manner in animals [139-141]. Importantly, these favorable effects on glycemic control and insulin regulation are closely associated with its anti-inflammatory and anti-oxidative effects. In HFD-fed animals who demonstrate insulin resistance and inflammation, green tea intake remarkably decreased pro-inflammatory cytokines TNFα, IL6, alleviated hepatic TLR4/ nuclear factor kappa B (NFκB) inflammation, increased glutathione and catalase levels, and decreased lipid peroxidation marker malondiadehyde in addition to lowering insulin resistance

and hepatic lipid levels [141-143]. In addition to downregulating inflammation in the periphery, green tea feeding was demonstrated to inhibit HFD-induced microglia activation in the arcuate nucleus of the hypothalamus, decrease inflammatory cytokine release, and suppress Janus kinase (JAK)2/signal transducer and activator of transcription (STAT)3 phosphorylation [144]. Recent evidence also suggests green tea mitigates HFD-induced obesity, inflammation, and gut microbiota alterations [143,145,146]. Taken together, green tea exerts various beneficial effects on obesity-related metabolic processes, including regulation of body weight, glycemic control, insulin sensitivity, lipid profile, oxidative stress, inflammation, and gut microbiota composition.

Bioavailability of Bioactive Compounds

From a pharmacological standpoint, bioavailability is the rate and extent to which the active ingredient or active moiety is absorbed from a bioactive compound or a drug and becomes available at the site of action [147]. It represents a pivotal factor determining the efficacy of bioactive compounds upon oral consumption that encompasses various stages such as liberation from a food matrix, absorption through the GI tract, distribution to the tissues, metabolism within the biological system, and elimination from the body [148]. Factors that may influence the bioavailability of bioactive compounds include the chemical structure of bioactive molecules, the transport mechanisms in the intestinal lumen, and the stability of bioactive compounds during metabolism by metabolizing enzymes, and interactions with other nutrients in the diet [149].

The bioavailability of some bioactive compounds is very low, which limits the use of bioactive compounds as functional ingredients [148]. One such example is phenolics, which are abundantly found in plant-derived foods such as hydroxycinnamates from coffee, flavan-3-ols in tea, and hesperidin from citrus fruits. Despite the potent health-promoting effects of polyphenols, the oral bioavailability of them is very low, ranging from 0.3% to 43% of the ingested dose [150].

As a result, the majority of polyphenols are cleared through the GI tract before making it into circulation [151]. Among the polyphenols, gallic acid and isoflavones have relatively higher bioavailability while galloylated tea catechins and anthocyanins are the least absorbed polyphenols [150].

Several approaches have been proposed to improve the bioavailability of bioactive compounds, such as nanotechnology that reduces particle size and modifies surface properties, encapsulation that enhance the delivery of bioactive molecules to active sites, modify the food processing procedures, or co-intake with other bioactive compounds that alters bioavailability through nutrient-nutrient interaction [148]. For example, catechin can increase the plasma concentration and cell uptake of puerarin, while puerarin decreased the plasma level and cell uptake of catechin [152]. These evidences imply the interactions between bioactive molecules that may modify their bioavailability when taken together.

Summary

The perinatal period represents a critical time of rapid neurocognitive development, with fast DHA playing a pivotal role in these processes. Epidemiological evidence suggests the neuro-supportive effects of DHA in brain development while mixed findings were reported with maternal and direct infant supplementation, urging further investigations into the roles of perinatal DHA consumption on cognitive performances and brain functional organization. On the other hand, DHA influences the gut microbiome composition and the gut-brain crosstalk through the HPA axis, inflammation, and neuroactive metabolites, which may, at least partly, contribute to the central regulation of DHA in the developing brain. Various bioactive compounds play a part in both central and systemic regulation. Considering green tea polyphenols exert beneficial effects against HFD-induced obesity, inflammation, insulin resistance, and dyslipidemia, it is

reasonable to speculate that green tea consumption will also mediate the adverse metabolic outcomes following HFD. Despite the health benefits of green tea polyphenols, their efficacy is limited by the low bioavailability, which may be enhanced through co-intake with other bioactive compounds. Therefore, this dissertation seeks to address three specific gaps in the current body of evidence: 1) the effects of perinatal DHA supplementation on the neurocognitive function in infants, 2) the roles of green tea in regulating obesogenic diet-induced metabolic dysfunction, and 3) the potentials of improving the bioavailability of green tea by co-intake with citrus juice.

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CHAPTER 3[1]

PERINATAL DOCOSAHEXAENOIC ACID SUPPLEMENTATION IMPROVES COGNITION AND ALTERS BRAIN FUNCTIONAL ORGANIZATION IN PIGLETS

1. Fang X, Sun W, Jeon J, et al. Perinatal Docosahexaenoic Acid Supplementation Improves Cognition and Alters Brain Functional Organization in Piglets. *Nutrients.* 2020;12(7). Reprinted here with permission of the publisher.

Abstract

Epidemiologic studies associate maternal docosahexaenoic acid (DHA)/DHA-containing seafood intake with enhanced cognitive development; although, it should be noted that interventional trials show inconsistent findings. We examined perinatal DHA supplementation on cognitive performance, brain anatomical and functional organization, and the brain monoamine neurotransmitter status of offspring using a piglet model. Sows were fed a control (CON) or a diet containing DHA (DHA) from late gestation throughout lactation. Piglets underwent an open field test (OFT), an object recognition test (ORT), and magnetic resonance imaging (MRI) to acquire anatomical, diffusion tensor imaging (DTI), and resting-state functional MRI (rs-fMRI) at weaning. Piglets from DHA-fed sows spent 95% more time sniffing the walls than CON in OFT and exhibited an elevated interest in the novel object in ORT, while CON piglets demonstrated no preference. Maternal DHA supplementation increased fiber length and tended to increase fractional anisotropy in the hippocampus of offspring than CON. DHA piglets exhibited increased functional connectivity in the cerebellar, visual, and default mode network and decreased activity in executive control and sensorimotor network compared to CON. The brain monoamine neurotransmitter levels did not differ in healthy offspring. Perinatal DHA supplementation may increase exploratory behaviors, improve recognition memory, enhance fiber tract integrity, and alter brain functional organization in offspring at weaning.

Keywords: omega-3 fatty acids; maternal nutrition; cognition; resting state network; brain development

Introduction

Prenatal and early postnatal time represent critical periods of brain development sensitive to nutritional status 1. Docosahexaenoic acid (DHA) is a n-3 long-chain polyunsaturated fatty acid (LCPUFA) commonly recommended during pregnancy and is widely added to the infant formula due to its suggested beneficial role in visual function and cognitive development 2,3. DHA accounts for more than 40% of the total n-3 PUFA in the neuronal tissue 4, especially in the gray matter 5,6. In the developing brain, the fastest DHA increase takes place during the third trimester of the gestation period, into the postnatal life 7,8, coinciding with the rapid myelination, dendritic outgrowth, and synaptogenesis 9, suggesting the importance of DHA during the critical window of neurodevelopment. Indeed, the perinatal deprivation of α-linolenic acid (ALA), the precursor of DHA, resulted in a 61% reduction in DHA concentration, 65% decrease in serotonin (5-HT) levels in the prefrontal cortex (PFC) 10, and disturbed hippocampal (HC)-dependent learning and cognitive behaviors in rats compared to offspring born to ALA-sufficient animals 11. Importantly, endogenous DHA synthesis is limited in mammals and breast milk serves as a high bioavailable source of DHA to infants 12.

In humans, compelling evidence from observational studies supports the positive association between maternal fish intake and infant DHA status in neurocognitive development 13-15. However, intervention trials supplementing DHA during gestation and/or lactation yielded mixed and conflicting findings in healthy term infants 16-20. For example, the Avon Longitudinal Study of Parents and Children (ALSPAC) found that maternal seafood intake higher than 340g/week is associated with better verbal intelligence quotient (IQ), decreased risk of suboptimal fine motor, social development, and communication scores of the children compared to those born to mothers who consumed less than 340 g seafood/week during

pregnancy 14. Nevertheless, a randomized control trial (RCT) in Australian women found that maternal supplementation of DHA (800mg/d) during pregnancy did not influence cognitive and language development of infants at 18 months compared to the control group 21. Similarly, DHA supplementation (220mg/d) during gestation until 3 months after delivery did not influence neurodevelopment of infants when they were evaluated at 18 months of age 22; fish oil supplementation (400ml/d) from 28th week of gestation to 4 month lactation did not influence visual and cognitive/psychomotor development of infants in their first year of life. These conflicting findings urge further exploration into the role of maternal DHA supplementation on the structural and functional organization of the developing brain in healthy subjects.

Pigs are a robust model for both nutritional and neurodevelopmental research in understanding nutritional programming in the human mother-infant dyad due to a myriad of similarities to humans in physiology, anatomy, pathology, and eating behavior 23-26. Pigs have a gyrencephalic brain 27,28, unlike mice who are lissencephalic, a key architectural difference that has a direct correlation with brain connectivity and complexity 29-32. The human and swine brain is composed of >60% white matter, while the white matter in the rodent brain is <10% 33,34. The anatomical similarities in different brain regions, such as the HC 35, subcortical and diencephalic nuclei 36, and cortical regions 37,38 between the pig and human brain have been illustrated. Furthermore, the pig brain exhibits a perinatal growth spurt much like human infants during which the brain weight grows most rapidly from the last trimester of gestation to lactation 39,40. Pig brain grows most rapidly from about 50 days pre- to 40 days postnatal 40; the cerebrum presents two rapid growth period between 80-100 days of conception and 6-26 days after birth 41. In healthy human infants, the brain volume is around 25% of adults' brain at birth and grows rapidly to around 72 and 83% of adults' total brain volume at 1 year and 2 years after

birth, respectively 42. Longitudinal MRI analysis in pigs revealed that the total brain volume reaches 75% and 95% around 10.7 and 22.07 weeks after birth, respectively 43.

This is the first study, to our knowledge, that thoroughly examines the effect of perinatal DHA intake on functional and cognitive development of the brain in piglets. The present study aims to investigate whether maternal supplementation of DHA during late gestation and lactation imparts advantages to cognitive development, fiber bundle maturation, anatomical and functional organization, and monoamine neurotransmitter status of the developing brain in healthy offspring using a piglet model.

Materials and Methods

Animals and Study Design

Cross-bred commercial line of healthy pregnant sows (n=8) were obtained from the University of Georgia Swine unit at approximately day 69 of gestation. Sows were assigned to the DHA group (DHA, n=5) or the isocaloric control group (CON, n=3) after accounting for the parity (5.38 ± 0.32) and body weight (BW, 239.7 ± 7.71 kg). After one week of acclimation in a temperature-controlled facility, sows were fed the corresponding diets from day 74 of gestation until delivery and throughout lactation. Approximately one week before expected farrowing, sows were transferred to individually housed farrowing crates equipped with heat lamps. From each litter, 2-3 male and female piglets with a BW closest to the average BW were chosen within 24 hours to be included in the study (n=14 in CON, n=20 in DHA). At weaning, piglets underwent behavior examinations, and a subset of piglets (n=7 in both CON and DHA) underwent MRI acquisition and were sacrificed at approximately the postnatal day (PND) 20 for tissue collection (Figure 1). This study was conducted per the University of Georgia Institutional Animal Care and Use Committee guidelines (project code: A2018 04-003-Y2-A8).

Dietary Treatment

Pregnant sows were maintained on a gestation (2 kg/d) and lactation (ad libitum) diet primarily composed of corn with a total of 3300-3330 kcal/kg as recommended by National Research Council (NRC), 2012 [44]. Sows in the DHA group were supplemented with 75 mg/kg BW/d DHA as algae-produced DHASCO (contains 42.9% DHA, DSM Nutritional Products Inc.) by mixing the oil with the basal diet (Table 1). Poultry fat and corn are the primary sources of fat in the basal diet. The major fatty acids in the basal diet, based on the fatty acid composition of poultry fat and corn [45,46], are linoleic acid (C18:2n6, LA, approximately 46% in gestation diet and 43% in lactation diet), oleic acid (18:1, approximately 33% in gestation diet and 34% in lactation diet), and palmitic acid (16:0, approximately 13% in gestation and 15% in lactation diet). The current dosage of DHA was chosen based on previous findings that effectively enhances DHA level in sows and piglets and exerts beneficial health effects on the animals [47,48]. To determine whether this dose is safe, liver alanine aminotransferase was measured using a clinical chemistry analyzer which confirmed that the current dose did not cause hepatotoxicity. Sows in the CON group were on an isocaloric diet mixing the basal diet (Table 1) with safflower oil (Jedwards International Inc.), which contains predominantly LA [49] that ensured no additional intake of ALA of the control animals.

Colostrum Fatty Acid Composition

Colostrum was collected within 12 hours postpartum from the functioning teats of the same position from all sows. The fatty acid concentration was measured using gas chromatography (Shimadzu, model 14 A, Tokyo, Japan) with a flame ionization detector as previously described [50]. Briefly, 2 ml colostrum samples were transmethylated according to the method of Park and Goins [51] and 2 mg of tridecanoic acid (C13:0) was added as an internal

standard before processing. The upper hexane layer was collected and residual water was removed by adding anhydrous sodium sulfate. Fatty acid methyl esters were separated on a Phenomenex, ZBWax Plus wide-bore capillary column (60 m × 0.53 mm, 1.00 μm film thickness; Phenomonex, Torrance, CA) with nitrogen as the carrier gas. Initial column temperature was 160 °C, temperature was held for 10 min and increased at a rate of 5 °C/min until 220 °C. Injector temperature was 250 °C and detector temperature was 260 °C. Peaks were identified by comparison of retention times of known standards (Nu-Chek Prep, Elysian, MN).

Behavior Testing

In order to habituate piglets to human touch and novel environments, all piglets were handled and habituated on a daily basis from PND2 until the day of behavior tests.

Open Field Test

As a measurement of ambulation and exploratory behaviors, piglets underwent the open field test (OFT) at approximately PND18 (PND 18.47± 0.097) in a 2.7 m x 2.7 m open arena lined with black mats. White curtains were hung around the arena to eliminate any visual distraction and the floor was sanitized with 70% ethanol between every trial to reduce olfactory bias. All piglets were exposed to the open arena for the first time. The piglets were individually introduced to the arena from the entry gate and allowed to explore for 10 min. Their exploratory behaviors (sniffing the wall), ambulation behaviors (mobile time, moving time), velocity, distance moved, and time spent in the center were recorded and measured by EthoVision video tracking software (Noldus, Wageningen, the Netherlands). The center zone size was designated as 0.9 m x 0.9 m in the center of the arena (Figure 2).

Object Recognition Test

As a measurement of memory retention, piglets underwent the object recognition test (ORT) which has been successfully applied in pigs [52]. The ORT was comprised of two trials: a sample trial and a test trial. After habituation to the arena in OF, the piglet was reintroduced to the arena with two identical objects attached in the center of the open arena and was allowed to explore both objects for 10 min. The sample trial was immediately followed by a 10-min interphase delay during which one object was replaced with a novel object. In the test trial, the piglet was introduced back to the arena and explored both the familiar and novel object for 10 min. All objects used in the study were cleaned thoroughly with water and ethanol between trials. The time that each piglet spent with each object in the sample and test trials were measured by Etho Vision (Noldus, Wageningen, the Netherlands). Proportional time was calculated as the ratio of time spent exploring the novel object to the total time exploring both objects in the sample and test trials.

MRI Acquisition

A subset of piglets (n=14) underwent MRI imaging at approximately PND20 (PND 20.07 ± 0.31). Piglets were sedated with propofol for intubation (0.083-0.166 ml/kg, IV) and maintained under mild anesthesia with 1.5% isoflurane during scanning. MRI scanning including T1-weighted anatomical, diffusion tensor imaging (DTI), and resting-state fMRI (rs-fMRI) were conducted at the Bioimaging Research Center at the University of Georgia utilizing a 3.0 Tesla General Electric (GE) HDx scanner and a quadrature knee coil. Piglets were monitored throughout the scan by a veterinary technician. A 3D fast spoiled gradient echo sequence (repetition time (TR) = 5.5 s, echo time (TE) = 2.1 ms, flip angle (FA) = 9°, field of view (FOV) = 12.8 × 12.8 × 6.4 cm, slice thickness = 1 mm, acquired matrix = 256 × 256 × 112) was used to acquire T1-weighted anatomical data; a spin-echo echo-planar imaging (EPI) sequence (TR =

15.5 s, TE = min-full, FOV = 12.8 × 12.8 × 6.4 cm, acquired matrix = 64 × 64 × 32, and 30 diffusion weighted images using b=1000s/mm2) was used for DTI acquisition; and rs-fMRI was acquired by a gradient-echo EPI sequence (TR = 3 s, TE = 30 ms, FA = 80°, FOV = 12.8 × 12.8 × 6.4 cm, acquired matrix = 96 × 96 × 32, a total volume of 300 images).

MRI Analysis

Anatomical and DTI Analysis

For anatomical MRI analysis, the individual brain was coregistered with a standard pig brain atlas [53] using the Statistical Parametric Mapping (SPM) toolbox. The percentage volume of 19 regions of interest were calculated using MATLAB (MATLAB R2018b, Natick, Massachusetts: The MathWorks Inc). For the DTI dataset, brain tissue was separated from the skull and other surrounding tissues by manual segmentation using 3D Slicer 4.11.0, and tractography (mapping of nerve fiber tracts) was performed using the Tensor Toolkit (TTK) for tensor estimation and tensor tractography using the software MedInria (https://med.inria.fr/). Whole brain tractography was performed with fibers seeding from voxels with FA values greater than approximately double the whole-brain average and stopping at voxels with FA values less than approximately two-thirds of the whole-brain average. A more detailed, standard pig brain atlas [54] was then used to coregister each individual DTI dataset using the SPM toolbox in MATLAB. Measures of mean diffusivity (MD), fractional anisotropy (FA), and fiber length (FL) were obtained for the fibers intersecting with the region of interest from the atlas.

Functional Connectivity Analysis

The rs-fMRI data (n=7 for each group) was analyzed by using sparse dictionary learning (sDL) [55], a machine learning approach that has successfully detected brain functional connectivity [56]. Specifically, fMRI time series was temporally concatenated to form a single

matrix X, which was decomposed into two matrices, i.e., X = D • α, where D is the dictionary matrix aiming to learn temporal patterns from the concatenated time series (each column referred to as an atom), and α is a weight matrix that indicates the weights of learned features in the dataset. By using a back propagation process the dictionary was iteratively learned, and the corresponding α-matrix were used to generate functional activation maps, corresponding to each atom.

Pearson correlation (PC) was calculated by using the α-matrix and an atlas of piglet brain [56]. Three atoms with the highest PC coefficients were selected for the group analysis, separately for six resting state network (RSN) including the executive control network (ECN), cerebellum network (CERE), visual network (VIS), sensorimotor network (SMN), auditory network (AUD), and default mode network (DMN).\

Animal Sacrifice and Tissue Collection

At weaning (PND 19.97 ± 0.14), all piglets were euthanized via CO2 asphyxiation. The brains were collected and coronally sectioned using a pig brain slicer (Zivic Instruments, Pittsburgh, PA) and fresh tissues of HC and PFC were collected and immediately frozen in liquid nitrogen and stored in -80 °C for future analysis.

HPLC-ECD

Monoamine neurotransmitters including dopamine (DA), serotonin (5-HT), and norepinephrine (NE) and their metabolites from PFC and HC were measured by the electrochemical detector for high-performance liquid chromatography (HPLC-ECD) as previously described [57,58]. Frozen tissue aliquots were mixed with 0.2 N perchloric acid (10 mg/100 μl), sonicated, and centrifuged. 20 μl of the supernatant was injected into HPLC to determine: (1) DA and DA metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic

acid (HVA); (2) 5-HT and its metabolite 5-hydroxyindoleacetic acid (5-HIAA); and (3) NE in brain tissues. The data are analyzed and presented as a ng of analyte per mg tissue.

Statistical Analysis

Data expressed as mean ± S.E. was analyzed using R (version 3.6.0) and GraphPad Prism (Version 7.00; GraphPad Software, Inc.; San Diego, CA, USA). The colostrum fatty acid composition from CON and DHA sows was analyzed using GraphPad Prism. For other data of piglets, a linear mixed-effect model was fitted to test the treatment while controlling the gender and maternal factors as fixed and random effects, respectively. The statistical significance of the treatment effect was determined based on t-statistics with the significance level of 0.05.

Results

Colostrum Fatty Acids Composition

The colostrum total lipids and lipid percentage did not differ in the two groups ($p > .05$, Table 2). Similar to the fatty acid profile in human colostrum, we detected high contents of palmitic acid (C16:0), oleic acid (C18:1), and LA (C18:2) [59]. Compared to CON, the DHA supplementation drastically increased the relative percentage levels of colostrum DHA (C22:6, 0.04 ± 0.01 and 3.36 ± 0.20 for CON and DHA, respectively, $p <.0001$) and other n-3 PUFA including eicosapentaenoic acid (EPA, C20:5, 0.04±0.02 and 0.48±0.06 for CON and DHA, respectively, $p = .001$) and docosapentaenoic acid (DPA, C22:5, 0.17±0.07 and 0.36±0.04 for CON and DHA, respectively, $p = .04$) without influencing ALA level (C18:2, 0.83 ± 0.03 and 0.80 ± 0.07 for CON and DHA, $p > .05$) (Table 2). Concomitantly, the relative percentage levels of colostrum n-6 PUFA were markedly reduced in sows fed a DHA diet including LA (C18:2, 35.98±2.03 and 27.55 ± 1.72 for CON and DHA, respectively, $p = .02$), dihomogammalinolenic acid (C20:3, 0.38 ± 0.05 and 0.23 ± 0.01 for CON and DHA, respectively, $p = .01$), and

arachidonic acid (AA, C20:4, 1.16 ± 0.08 and 0.46±0.01 for CON and DHA, respectively, $p < .0001$) (Table 2). Several other minor fatty acids including lauric acid (C12:0), myristic acid (C14:0), myristoleic acid (C14:1), and palmitoleic acid (16:1) were also elevated in DHA-fed sows compared to CON (Table 2).

Piglet Bodyweight and Brain Weight

Piglets selected for this study were born with BW 1450.10 ± 68.84 g and grew to 5939.03 ± 288.00 g before weaning with an average daily weight gain of 223.48 ± 11.77 g/d and a final brain weight of 49.22 ± 0.63 g (averages of the two groups combined). Maternal supplementation of DHA and the isocaloric CON diet from late gestation to lactation did not influence the birth weight, final BW, weight gain, daily weight gain, and brain weight of the piglets at the end of the study (Table 3).

Behavior Testing

Open Field Test

There was no statistically significant difference in the total distance moved, velocity, mobile time of any body part, and moving time of the body center point between CON and DHA piglets. This suggests that maternal DHA supplementation did not influence the locomotor function of healthy offspring (Figure 3A-D). Similarly, piglets from the two groups spent a comparable amount of time and similar visiting frequency in the center zone of the arena (Figure 3E-F). Interestingly, the piglets born to DHA-fed sows spent 95% more time sniffing the walls of the open arena compared to CON (p=.002, Figure 3G-H), demonstrating that the maternal DHA supplementation may enhance the inquisitive and exploratory behavior of the healthy offspring without affecting the normal development of motor function at weaning.

Object Recognition Test

During the sample trial as explained in the methods section, all piglets spent similar time and frequency exploring the two identical objects with no preference ($p>.05$). In the test trial, male and female piglets born to CON-fed sows spent similar proportional time and frequency (Figure 4A-D) exploring the familiar and novel objects ($p > .05$). Male piglets from DHA-fed sows showed a trend to spend more proportional time with the novel object ($p=.060$, Figure 4E), but not in proportional frequency (Figure 4F). The female piglets born to DHA-fed sows exhibited significantly more interest in the novel object than the familiar object as they spent more proportional time ($p=.032$, Figure 4G) and proportional frequency ($p=.008$, Figure 4H) engaging with the novel object. This indicates that maternal DHA supplementation may improve hippocampal-dependent short-term learning and memory of the offspring.

Structural MRI Analysis

The volumes of different brain regions in piglets born to CON and DHA-fed sows at weaning are presented in Table 4. At approximately PND 20, MRI volumetric assessments showed that piglet brains were composed of 63.4% cortex, 11.39% cerebellum, 2.76% thalamus, 1.84% HC, and 4.63% olfactory bulb and other subcortical regions. The maternal intake of DHA during late gestation and lactation did not pose any prominent change in brain volumes of these healthy offsprings. Although we found a statistically significant 0.0035% decrease in the thalamus, the clinical significance of this observation is unclear. In addition, the left and the right cortex tended to be slightly larger in male subjects compared to that of female subjects (left cortex: 31.2615 % and 31.2565% for male and female, respectively; right cortex: 32.1408 % and 32.1493 % for male and female, respectively, $p < .07$). Overall, DHA supplementation during normal pregnancy and lactation did not result in significant changes in brain structural development in healthy offspring.

Hippocampal DTI Analysis

Axons insulated by a myelin sheath and firmly packed axonal bundles are critical for neuronal signal transduction and information processing which may contribute to improved cognitive performance. Whole-brain tractography was performed in piglets born to CON- and DHA-fed sows (Figure 5A1-A2). Tract-based DTI analysis showed that DHA supplementation during gestation and lactation did not influence MD in piglet HC ($p > .05$, Figure 5B1). However, there was a trend of higher FA values in piglets born to DHA-fed sows (0.21 ± 0.003 and 0.23 ± 0.005 for CON and DHA, respectively, $p = .07$, Figure 5B2). Additionally, we observed a significant increase of FL in the HC of piglets born to DHA-fed sows (27.02 ± 0.93 mm) compared to piglets born to CON-fed sows (19.84 ± 2.07 mm, $p = .01$, Figure 5B3).

Functional Connectivity Analysis

In order to test whether maternal supplementation of DHA influences the large-scale cortical networks, we examined neural network organizations in six identified RSNs (Table 5). Earlier, using sDL, we successfully detected these networks in piglets that resemble their counterparts in human brains [56]. PC analysis was performed using the sDL activation maps and RSN atlas [56]. The PC analysis results and representative brain activation maps are presented in Figures 6 and 7. Perinatal DHA supplementation resulted in an 8.7% increase of functional connectivity within CERE ($r = 0.3818$ and 0.4152 for CON and DHA, respectively, Figure 6B/7B), 5.2 % enhanced connectivity within VIS ($r = 0.3915$ and 0.4120 for CON and DHA, respectively, Figure 6C/7C), 9.8% increase within DMN ($r = 0.3048$ and 0.3346 for CON and DHA, respectively, Figure 6F/7F) and a minor increase within AUD ($r = 0.2523$ and 0.2526 for CON and DHA, respectively, Figure 6E/7E) compared to CON piglets. In addition, piglets born to DHA-fed sows showed a 7.1% decrease in ECN ($r = 0.5217$ and 0.4849 for CON and DHA,

respectively, Figure 6A/7A) and a 8.9% decrease in functional connectivity in the SMN compared to that of CON piglets (r = 0.3149 and 0.4120 for CON and DHA, respectively, Figure 6D/7D).

Monoamine Neurotransmitters

In order to determine if the effects of DHA is through the regulation of monoamine neurotransmission, we measured key monoamine neurotransmitters and their metabolites using HPLC (Table 6). In the PFC, the levels of DA, 5-HT, NE and their metabolites did not differ between piglets born to CON and DHA-fed sows ($p > .05$). Piglets from DHA-fed sows had 32.94 % higher DA, 30.48 % higher 5-HT, and 45.83 % higher NE in the HC relative to that of CON piglets; however, none of these reached statistical significance, likely due to the small sample size. Metabolites of DA and 5-HT in HC were not different between the two groups.

Discussion

DHA is commonly recommended for pregnant women and is widely added in infant formula for its potential benefits in visual and brain functions 60,61. However, interventional trials have yielded conflicting neurocognitive outcomes. In this study, we found that maternal DHA supplementation increased exploratory behaviors, short-term object recognition memory, fiber length in the HC, and enhanced functional connectivity in key brain networks of healthy offspring.

We found that DHA supplementation during late gestation and lactation resulted in a remarkably 80-fold increase in the colostrum DHA concentration along with the other n-3 PUFA without influencing the ALA level. This is in agreement with the previous findings from humans and pigs, that maternal dietary intake of DHA sufficiently modified breast milk DHA and other n-3 PUFA such as EPA (C20:5) and DPA (C22:5) levels while ALA levels remained relatively

stable 62-66. Although we did not measure plasma DHA level in the offspring, other studies have shown that supplementing lactating mothers with DHA, increased infant plasma and erythrocytes phospholipid DHA levels 64,65. Meanwhile, breastmilk n-6 PUFA such as LA (C18:2) and AA (C20:4) were decreased due to dietary DHA supplementation in accordance with previous findings in humans that these n-6 PUFA in breast milk and n-6/n-3 ratio in infant plasma were reduced by DHA supplementation during pregnancy and lactation likely due to the competitive incorporation into the plasma membrane between the two 66,67.

Piglets born to DHA-fed sows demonstrated better cognitive performance in the behavioral testing. The OFT provides a simple and general measure of motor function and exploratory behaviors 68 and the ORT measures short-term object recognition memory, which is at least partly hippocampus-dependent 69 in an enclosed and undisturbed setting. Previous studies demonstrated selective hippocampal lesion led to impaired object recognition memory in both human and non-human primates 70,71, indicating a critical role of hippocampus in the short-term recognition memory. Additionally, the development of memory ability is suggested to be dependent partly on the progressive development of the hippocampus in a sequence of novelty preference, cognitive recall, flexible memory, and source memory 72. Healthy offsprings from DHA-fed and CON-fed sows exhibited similar locomotor functions. Interestingly, piglets from DHA-fed sows demonstrated more exploratory behaviors as they spent more time sniffing the walls of the open arena. Dietary intake of n-3-PUFA was found to be associated with increased exploratory behaviors in other models 73,74. This may be due to decreased stress and anxiety levels and/or increased visual function of the piglets from the DHA group. In rodents, a low intake of n-3 PUFA reduced exploratory behaviors in young animals and dietary DHA decreases stress and anxiety levels 74-76. In pigs, postnatal DHA deprivation depleted frontal cortex DHA

and increased fear/anxiety-like behavior associated with brain DA levels 77. The improved recognition memory formation assessed by ORT is in agreement with previous findings in which preterm piglets supplemented with DHA had increased recognition memory compared to the control 78. The fact that the control piglets did not have a preference for the novel object at this stage, indicates that a regulatory recommendation for DHA supplementation during neurodevelopment is worthwhile considering. Nevertheless, more data and studies are needed as the memory-improving effect of DHA is widely seen in young adults 79 but not consistently observed in infants 20,80,81. Noteworthy, these human trials commonly used the Fagan Test of Infant Intelligence to assess the recognition memory with only visual stimuli 82. In comparison, the ORT in animals provides the subjects an opportunity to explore the objects with physical contact. The enhanced exploratory behavior and memory may also be related to the improved visual acuity due to DHA treatment. Human infants fed with a DHA-enriched diet showed accelerated development and maturation of the visual system and better visual function 83,84. We also found increased functional connectivity within the visual network in these piglets, suggesting that the increased curiosity to the surrounding environment and higher engagement during object recognition may be partly due to the advanced development of the visual system.

MRI such as structural MRI, DTI, and rs-fMRI is increasingly used as a powerful tool to evaluate the role of DHA in the cognitive development of preterm infants. In our study, the perinatal DHA supplementation increased the myelination of axonal bundles in piglets. DTI measures brain microstructure and has been largely used to assess white matter integrity indicated by a reduced FA value 85,86. Children born with very low birth weight had lower FA values in internal and external capsules, corpus callosum, and inferior and superior fasciculus than the control group which was associated with visual-motor deficits and lower IQ scores 87.

A lower FA score was found to be associated with language processing, reading skills, and attention and anxiety behaviors later in life of preterm-born children, and such relation was not observed in full-term infants 88,89. Breastfed infants have higher brain DHA concentrations 90, and preterm infants fed exclusively with breastmilk demonstrated greater structural connectivity and higher FA in major white matter fasciculi compared to non-exclusively breastfed infants 91. Moreover, postnatal DHA supplementation to premature infants for 9 weeks showed a trend of higher FA value in the corpus callosum related to control preterm infants at 8 years of age 92. In this study, DTI analysis revealed an increased fiber tract length and a trend of increased FA value, which is in agreement with the previous findings in which higher DTI indices in the HC was seen in DHA-fed preterm pigs 78.

Resting-state fMRI assesses intrinsic functional connectivity between different neural networks by measuring fluctuations in blood oxygen level-dependent (BOLD) signal while the subject remains at a resting state without doing any cognitive task 93. To the best of the authors' knowledge, this is the first study investigating the intrinsic RSN changes due to maternal intake of DHA during gestation and lactation. CERE is associated with various fundamental functions including motor coordination, visuomotor learning, executive function, and memory formation 94,95. Higher activity of CERE due to perinatal DHA supplementation is likely to benefit the visual guidance of movement and working memory coinciding with our observations in the behavioral testing. DMN represents the intrinsic and spontaneous neuronal activity associated with internal thought processes and is deactivated during cognitively demanding tasks 96,97. Failure of the DMN deactivation is associated with cognitive abnormities 98-100. While it is unclear why perinatal DHA supplementation increased DMN activity in piglets, the intervention is likely to pose long-term influences on the cognitive processing and neurodegenerative

processes 101. Further investigations of maternal DHA supplementation on DMN activity comparing resting and task-based fMRI in infants will be of interest.

An increased VIS activity with DHA supplementation may indicate the accelerated maturation of the visual system which underlies the improved visual acuity observed in human trials 83,84,102. Term infants are born with adult-like VIS and SMN networks, suggesting prenatal development within these two domains 103. We also found a decreased activity within SMN in piglets born to DHA-fed sows which may suggest that DHA prevents the activation of SMN at the resting state. Interestingly, in human infants, the rapid development of SMN may occur earlier than VIS network 104 and the within-network connectivity of SMN manifests an age-related decrease during the first two years 103; this may indicate an increased synaptic and axonal pruning within the sensory and motor cortices and a shift of functional organization towards more specialized cortical networks 105. Thus, a decreased functional connectivity within SMN may suggest an enhanced development and maturation of the network that might have resulted from maternal DHA supplementation. Our findings suggest that perinatal DHA supplementation may alter brain functional organization of the offspring and support rs-fMRI as a sensitive tool to assess DHA status on brain functional connectivity and cortical organization in healthy piglets.

Monoamine neurotransmitters are critical neurochemicals for the proper function of learning, memory, and emotions 106. Dietary n=3-PUFA deficiency drastically decreased DA level and increase 5-HT2 receptor in the PFC of young animals 107. In piglets, postnatal DHA and AA supplementation to ALA/LA deficient animals increased DA, 5-HT, and NE in the frontal cortex 108. Perinatal ALA deficiency decreased tyrosine hydroxylase level, the rate-limiting enzyme for DA synthesis, in the substantia nigra and ventral tegmental area of the

dopaminergic pathway 109 and increased 5-HT turnover in the PFC in rats 10. We found that during a healthy pregnancy, maternal DHA supplementation resulted in an insignificant increase in DA, 5-HT, and NE in HC of the offspring. It will be of interest to determine whether perinatal DHA intake influences neurotransmitters with larger sample size.

This is the first study to provide evidence for improvement in cognition and brain development with perinatal DHA supplementation in piglets. However, there are few limitations to this study. A small sample size, though statistically enough, may have limited our power to detect the true effect of perinatal DHA supplementation in piglets and increased the error due to the random effects including sow effects. The maternal factor was controlled in statistical analysis by including sow as a random effect using the mixed-effect linear model. Additionally, the present study used safflower oil as a control oil, which is high in n-6 PUFA. Thus, the observed changes may be ascribed in part to the high n-6 intake in the control animals. Although n-6 rich components like safflower oil are commonly used in the control diets to match the caloric intake between the control and test groups, safflower oil is low in oleic acid, which is the main fatty acid of myelin and could potentially influence myelination and brain functional connectivity during development 110. Finally, the current study measured fMRI when the animals were at resting state. While it provided valuable insights into the resting-state networks influenced by the treatment, it is challenging to interpret due to its novel application in infant piglets. Future studies integrating both resting-state and task-based fMRI in a larger sample size would be of interest.

Conclusions

In conclusion, the present data provides preclinical support that the maternal DHA supplementation during late gestation and lactation may increase exploratory behavior, improve

memory function, enhance fiber tract integrity, and alter brain functional organization of offspring at weaning without affecting the volume of major brain structures.

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Table 3.1. Diet composition of the basal diet during gestation and lactation.

Ingredients, g/kg	Gestation Diet[1]	Lactation Diet[1]
Corn	535.4	389.6
Soybean Meal	32.3	172.3
Distillers Dried Grains with Solubles	400.0	400.0
Fat	-	4.6
Dicalcium Phosphate	2.8	-
Limestone	17.4	21.6
Salt	3.5	3.5
Vitamin pre-mix[2]	2.5	2.5
Trace Mineral pre-mix[3]	1.5	1.5
Sow Vitamin pre-mix[4]	2.5	2.5
L-Lysine HCl	2.1	2.0
Total	1000.0	1000.0
Calculated Analysis		
Crude Protein, %	17.4	22.4
Metabolizable Energy, kcal/kg	3330	3300
Crude Fiber, %	4.18	5.10
Ether Extract, %	6.23	6.14
Lysine, %	0.70	1.10
TSAA, %	0.68	0.86
Threonine, %	0.64	0.92
Tryptophan, %	0.15	0.25
Ca, %	0.79	0.90
Total P, %	0.52	0.57
Available P, %	0.29	0.40

[1] Basal diet was supplemented with 75 mg/kg BW/d DHA as DHASCO or an equivalent amount of safflower oil daily in DHA and CON group, respectively.[2] Supplied per kg of premix: vitamin A 4,400 IU; vitamin D 660,000 IU; vitamin E 17,600 IU; vitamin K 1,760 IU; riboflavin 3,960 mg; niacin 22,000 mg; vitamin B12 17,600 μg. [3] Supplied per kg of premix: iron 110,000 mg; copper 11,000 mg; manganese 26,400 mg; zinc 110,000 mg; iodine 198 mg; selenium 198 mg.[4] Supplied per kg of premix: Biotin, 88 mg; Choline, 220.5 g; Folic acid, 661.5 mg; Pyridoxine, 1.98g; Vitamin E8,882 IU.

Table 3.2. Colostrum fatty acids composition from sows fed with/without DHA during late gestation and lactation.

	Control (n=3)	**DHA (n=5)**	***p*-value**
lipid	3.90±0.90	3.83±0.55	0.94
C12:0	0.04±0.00	0.09±0.00	0.01
C14:0	1.23±0.09	2.29±0.17	0.004
C14:1	0.02±0.00	0.07±0.01	0.01
C15:0	0.11±0.01	0.11±0.01	0.69
C16:0	18.31±0.69	19.50±0.50	0.20
C16:1	3.25±0.07	4.43±0.34	0.04
C17:0	0.30±0.01	0.24±0.01	0.01
C17:1	0.23±0.01	0.24±0.02	0.90
C18:0	4.70±0.52	4.30±0.32	0.51
C18:1	29.80±0.81	32.18±1.42	0.19
C18:2	35.98±2.03	27.55±1.72	0.02
C18:3n-6	0.64±0.08	0.39±0.05	0.03
C18:3n-3	0.83±0.03	0.80±0.07	0.78
C20:0	0.18±0.07	0.14±0.03	0.65
C20:1	0.34±0.03	0.37±0.02	0.45
C20:2	0.74±0.04	0.66±0.03	0.14
C20:3	0.38±0.05	0.23±0.01	0.01
C20:4	1.16±0.08	0.46±0.01	<0.0001
C20:5	0.04±0.02	0.48±0.06	0.001
C22:2	0.59±0.35	0.64±0.04	0.84
C22:5	0.17±0.07	0.36±0.04	0.04
C22:6	0.04±0.01	3.36±0.20	<0.0001

Relative percent of each fatty acid is shown. Data are presented as means ± S.E. Abbreviations: DHA: docosahexaenoic acid.

Table 3.3. Body weight and brain weight of piglets at weaning.

	Control (n=14)	DHA (n=20)	*p*-value
Birth weight (g)	1539.00±75.56	1389.00±103.10	0.67
Final weight (g)	6134.00±353.60	5790.00±436.40	0.73
Weight gain (g)	4528.00±338.00	4333.00±308.70	0.78
Daily weight gain (g/day)	229.30±16.87	219.80±16.28	0.77
Brain weight (g)	49.28±0.69	49.17±0.97	0.77

Data are presented as means ± S.E. A linear mixed-effect model was used to control for gender (fixed) and maternal (random) effects. Abbreviations: DHA: docosahexaenoic acid.

Table 3.4. Structural MRI analysis of nineteen brain regions of piglets at weaning.

Brain region	Control (n=7)	DHA (n=7)	*p*-value
Caudate	0.860±0.001	0.861±0.001	0.46
Cerebellum	11.389±0.002	11.388±0.001	0.71
Left Cortex	31.262±0.002	31.257±0.002	0.09
Right Cortex	32.143±0.002	32.144±0.001	0.54
Lateral Ventricle	1.012±0.002	1.014±0.0001	0.95
Third Ventricle	0.106±0.0003	0.105±0.0005	0.77
Cerebral Aqueduct	0.074±0.0003	0.074±0.0002	0.63
Fourth Ventricle	0.099±0.0005	0.100±0.0002	0.33
Left Hippocampus	0.914±0.0005	0.916±0.0006	0.15
Right Hippocampus	0.925±0.001	0.926±0.001	0.50
Medulla	3.364±0.002	3.3620±0.001	0.54
Midbrain	3.414±0.001	3.412±0.001	0.31
Pons	2.154±0.001	2.155±0.001	0.44
Putamen and Globus Pallidus	0.731±0.001	0.731±0.0004	0.78
Hypothalamus	0.501±0.001	0.502±0.001	0.13
Thalamus	2.771±0.001	2.767±0.001	0.04
Olfactory Bulb	4.632±0.001	4.634±0.002	0.48
Corpus Callosum	0.776±0.002	0.775±0.001	0.86
Internal Capsule	2.875±0.002	2.877±0.001	0.55
Total (voxel)	411301±11.94	411342±25.47	0.23

Percentage volume of different brain region is shown. Data are presented as means ± S.E. A linear mixed-effect model was used to control for gender (fixed) and maternal (random) effects. Abbreviations: DHA: docosahexaenoic acid.

Table 3.5. Resting-state network included in the analysis and their regions of interest.

Resting-state Network	Locations of Regions of Interest
Executive control network (ECN)	Primary somatosensory cortex
	Dorsolateral prefrontal cortex
	Anterior prefrontal cortex
	Orbitofrontal cortex
	Insular cortex
	Ventral anterior cingulate cortex
	Dorsal anterior cingulate cortex
Cerebellar network (CERE)	Cerebellum
Visual network (VIS)	Primary visual cortex
	Secondary visual cortex
	Associative visual cortex
Sensorimotor network (SMN)	Primary motor cortex
	Somatosensory association cortex
	Premotor cortex
Auditory network (AUD)	Superior temporal gyrus
	Auditory cortex
Default mode network (DMN)	Hippocampus
	Anterior prefrontal cortex
	Orbitofrontal cortex
	Inferior temporal gyrus
	Ventral posterior cingulate cortex
	Retrosplenial cingular cortex
	Dorsal posterior cingular cortex
	Anterior entorhinal cortex
	Parahippocampal cortex

Table 3.6. Concentrations of monoamines and their metabolites in PFC and HC in piglets at weaning.

Brain regions	Neurochemicals	Control (n=7)	DHA (n=7)	*P*-value
PFC	DA	3.463±0.390	3.551±0.454	0.85
	DOPAC	0.090±0.012	0.078±0.015	0.64
	HVA	0.121±0.015	0.092±0.009	0.61
	5-HT	0.174±0.022	0.124±0.030	0.08
	5-HIAA	0.067±0.010	0.050±0.010	0.25
	NE	0.168±0.022	0.163±0.039	0.89
HC	DA	2.459±0.181	3.269±0.371	0.14
	HVA	0.170±0.035	0.155±0.024	0.99
	5-HT	0.105±0.017	0.137±0.024	0.26
	5-HIAA	0.096±0.004	0.113±0.019	0.36
	NE	0.072±0.008	0.105±0.014	0.11

Data are presented as means ± S.E; unit: ng/mg protein. Abbreviations: DHA: docosahexaenoic acid; PFC: prefrontal cortex; HC: hippocampus; DA: dopamine; DOPAC: dihydroxyphenylacetic acid; HVA: homovanillic acid; 5-HT: serotonin; 5-HIAA: 5-hydroxyindoleacetic acid; NE: norepinephrine; A linear mixed-effect model was used to control for gender (fixed) and maternal (random) effects.

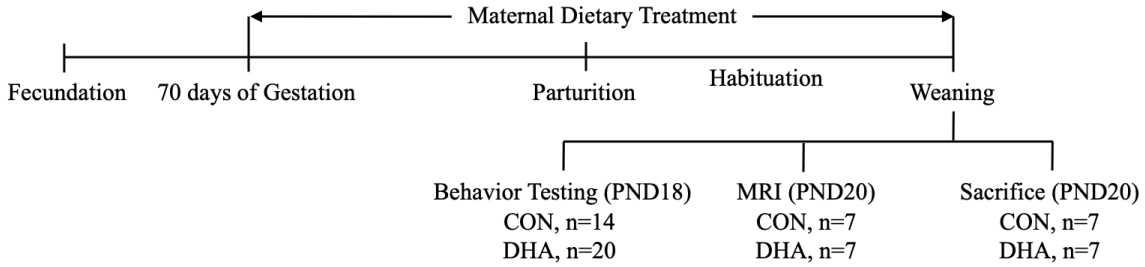

Figure 3.1. Study design and experimental timeline. CON: control; DHA: docosahexaenoic acid.

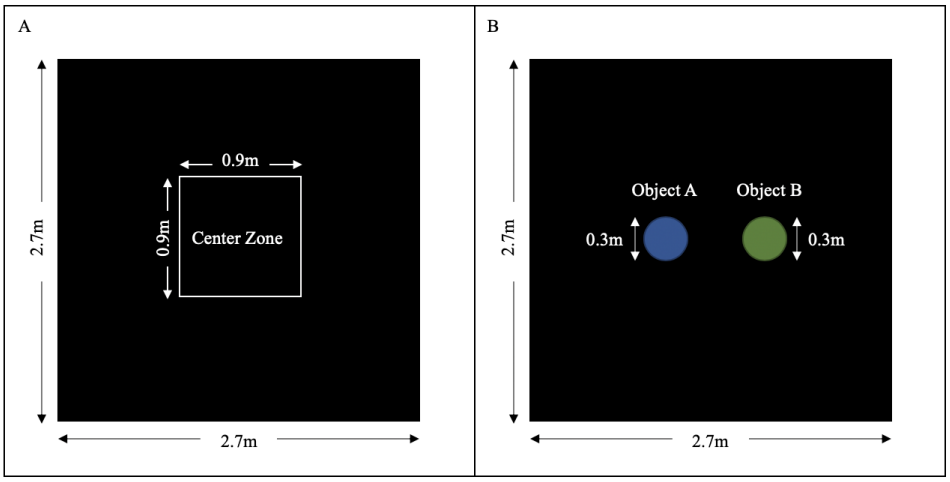

Figure 3.2. Schematic illustration of the testing arena for open field test (A) and the object recognition test (B).

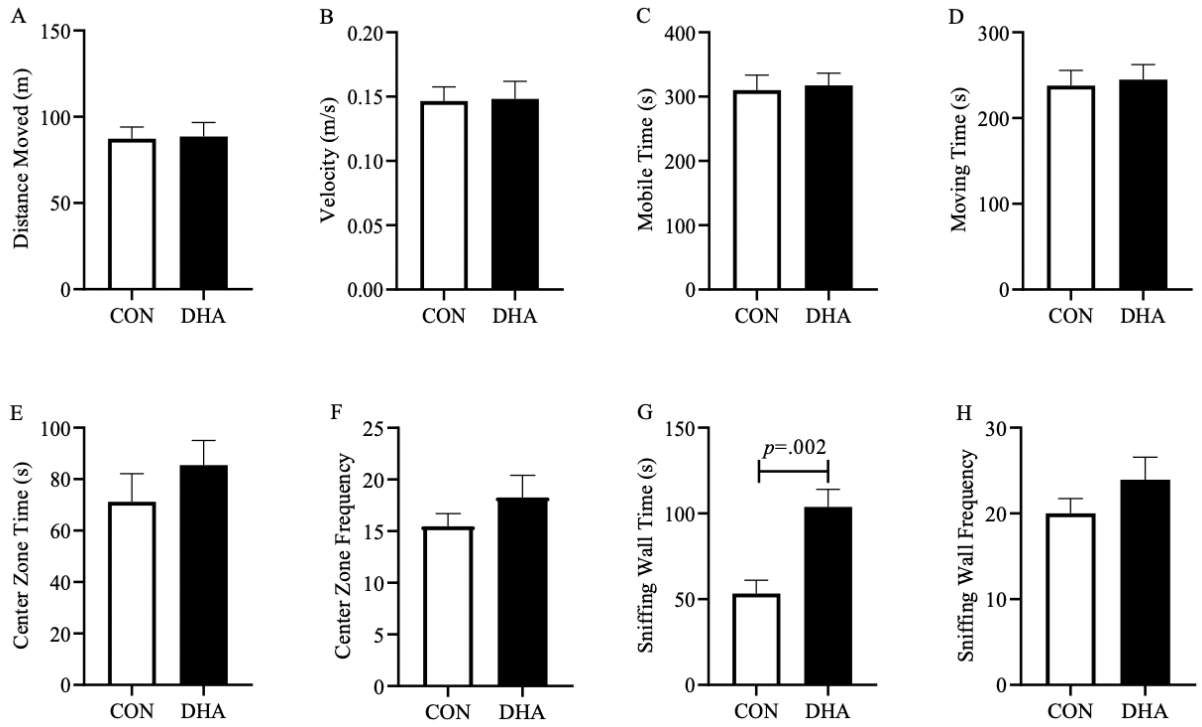

Figure 3.3. Measures of activity in the open field test for piglets born to sows fed a CON (n=14) or DHA (n=20) diet at weaning. A. distance moved; B. velocity; C. mobile time of any part of the body; D. moving time of the center point; E. time spent in the center zone; F. frequency visit the center zone; G. sniffing wall time; H. sniffing wall frequency. A linear mixed-effect model was used to control for gender (fixed) and maternal (random) effects. Abbreviations: CON: control; DHA: docosahexaenoic acid. *p* values higher than 0.1 are not shown on the graph.

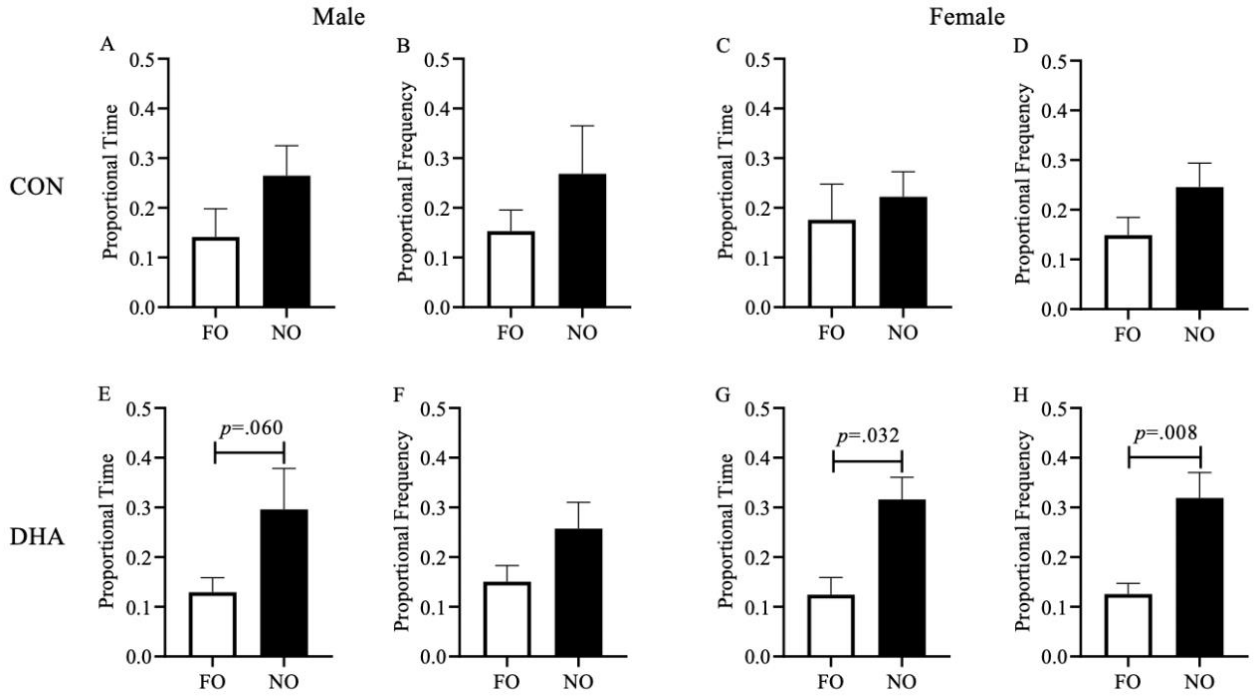

Figure 3.4. Measures of activity in the test trial of novel object recognition test for piglets born to sows fed a CON (n=14) or DHA (n=20) diet at weaning. Figures depicting proportional time and visit frequency on the familiar and novel objects. A. proportional time of control male piglets; B. proportional frequency of control male piglets; C. proportional time of control female piglets; D. proportional frequency of control female piglets; E. proportional time of DHA male piglets; F. proportional frequency of DHA male piglets; G. proportional time of DHA female piglets; H. proportional frequency of DHA female piglets; A linear mixed-effect model was used to control for gender (fixed) and maternal (random) effects. Abbreviations: FO: familiar object; NO: novel object. p values higher than 0.1 are not shown on the graph.

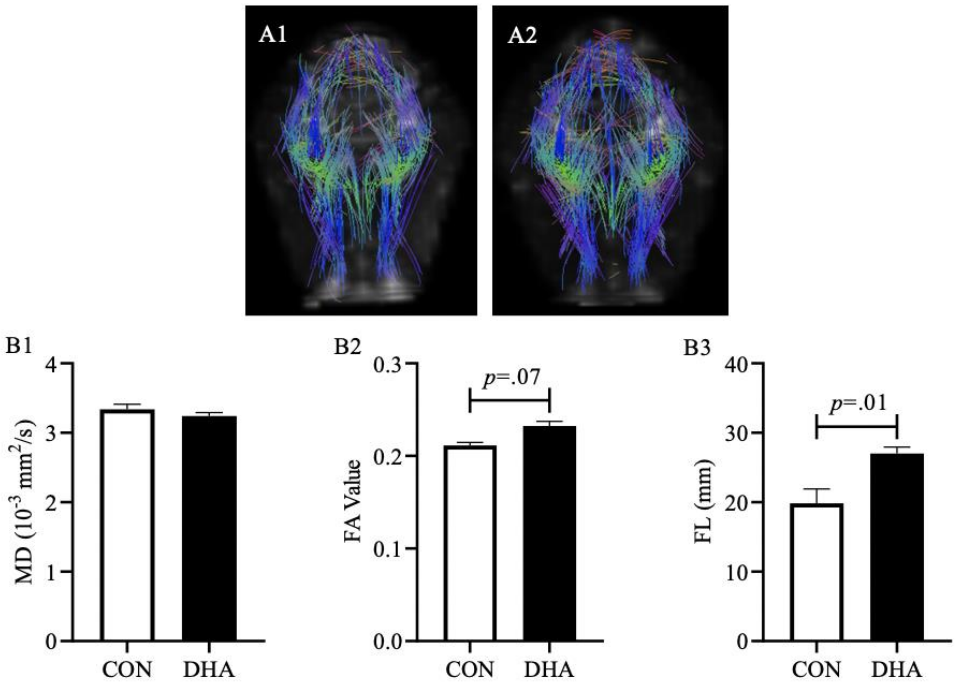

Figure 3.5. Diffusion tensor imaging analysis. Representative whole brain tractography of piglets born to sows fed a CON (A1, n=7) or DHA (A2, n=7) diet during late gestation and lactation. Mean diffusivity (B1), fractional anisotropy (B2), and fiber length (B3) within hippocampus of piglets. A linear mixed-effect model was used to control for gender (fixed) and maternal (random) effects. Abbreviations: CON: control; DHA: docosahexaenoic acid; MD: mean diffusivity; FA: fractional anisotropy; FL: fiber length. *p* values higher than 0.1 are not shown on the graph.

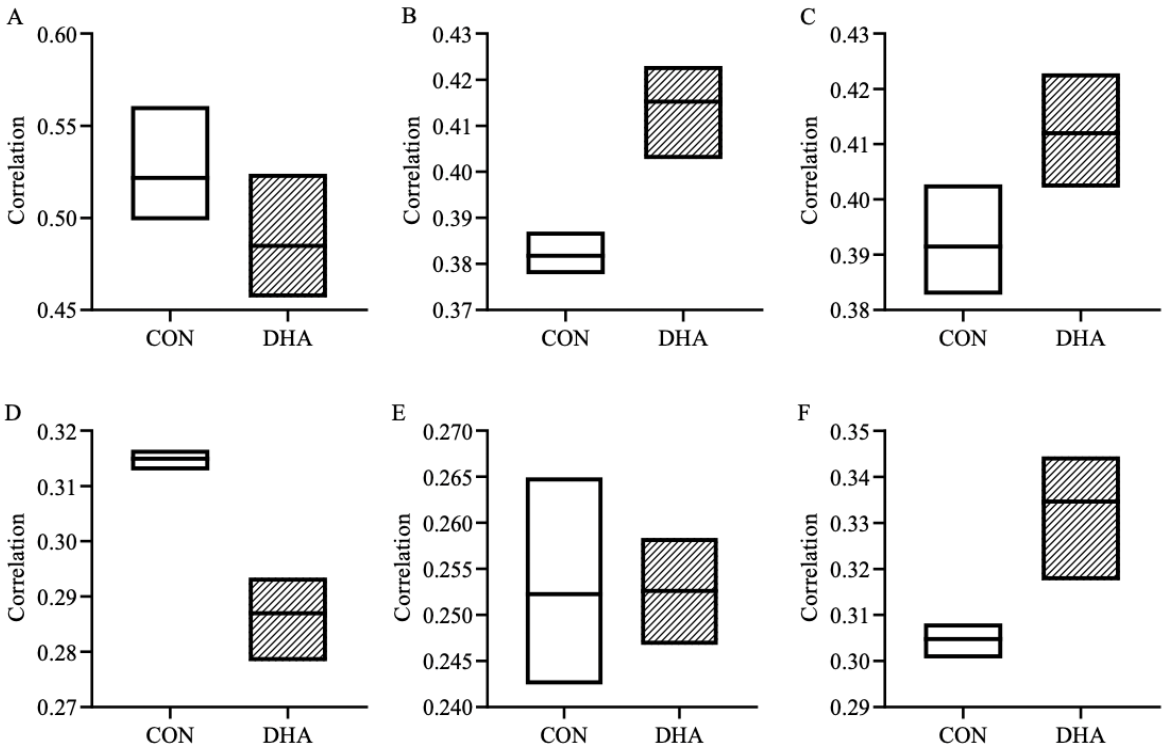

Figure 3.6. Group analysis of functional connectivity from rs-fMRI scan in six resting state networks including ECN (A), CERE (B), VIS (C), SMN (D), AUD (E), and DMN (F) in piglets born to sows fed a CON (n=7) or DHA (n=7) diet. Graphs show the correlation coefficients from the best three activation maps. Abbreviations: CON: control; DHA: docosahexaenoic acid. RSN: resting state network; ECN: executive control network; CERE: cerebellar network; VIS: visual network; SMN: sensorimotor network; AUD: auditory network; DMN: default mode network.

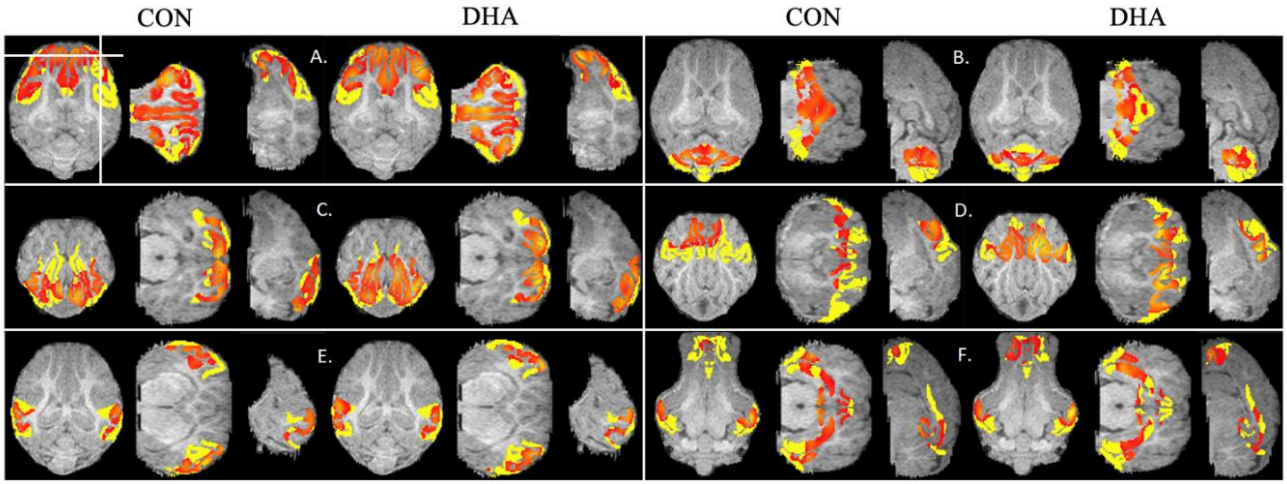

Figure 3.7. Color-coded regions illustrated activation within six RSN including ECN (A), CERE (B), VIS (C), SMN (D), AUD (E), and DMN (F) in piglets born to sows fed a CON (n=7) or DHA (n=7) diet. Each session contains activation maps of CON (left) and DHA (right) piglets in axial, coronal, and sagittal planes. Horizontal and vertical bars on the axial image at the upper-left corner indicate locations of the coronal and sagittal images. Yellow patters are RSN atlases and orange patters are activations. Abbreviations: CON: control; DHA: docosahexaenoic acid. RSN: resting state network; ECN: executive control network; CERE: cerebellar network; VIS: visual network; SMN: sensorimotor network; AUD: auditory network; DMN: default mode network.

CHAPTER 4[1]

DEPLETION OF REGULATOR OF G-PROTEIN SIGNALING-10 IN MICE EXAGGERATES HIGH-FAT DIET-INDUCED INSULIN RESISTANCE AND INFLAMMATION, AND THIS EFFECT IS MITIGATED BY DIETARY GREEN TEA EXTRACT

1. Fang X, Chung J, Olsen E, et al. Depletion of regulator-of-G-protein signaling-10 in mice exaggerates high-fat diet-induced insulin resistance and inflammation, and this effect is mitigated by dietary green tea extract. *Nutr Res.* 2018. Reprinted here with permission of the publisher.

Abstract

The interaction between insulin resistance and inflammation plays a central role in the development of chronic diseases with the mechanism not fully understood. We previously demonstrated regulator of G-protein signaling-10 (RGS10) protein as a negative modulator of the inflammatory response in macrophages and microglia. In this study, we aimed to investigate if RGS10 is involved in the regulation of glucose tolerance and insulin sensitivity that contribute to developing chronic diseases and conditions. We hypothesized that the absence of RGS10 will exaggerate high-fat diet (HFD)-induced insulin resistance and inflammation response. Our results showed that RGS10 knockout (KO) mice fed a HFD gained significantly more weight and developed severe insulin resistance compared to wild-type (WT) mice the fed HFD. Also, KO mice fed the HFD displayed inflammatory phenotypes compared to their counterparts by a decrease in the expression of YM1 and Fizz1 and an increase in the expression of interleukin 6 in adipose and liver tissues, which are an anti-inflammatory M2 marker and proinflammatory M1 cytokine, respectively. The impact of RGS10 deficiency on the exaggeration of HFD-induced insulin resistance and inflammation was ameliorated by oral consumption of GTE. Our result implicates a protective mechanism of RGS10 in regulating metabolic homeostasis by reducing inflammatory responses, which could potentially lead to an innovative new approach targeting inflammation and insulin resistance.

Keywords: RGS; Obesity; Glucose tolerance; Phytochemicals; Antioxidants

Introduction

Obesity is an increasing concern with a prevalence of 36.5 % among U.S adults according to the National Health and Nutrition Examination Survey in 2011-2014. Often accompanied by elevated systemic inflammation and impaired insulin sensitivity, obesity and its related metabolic syndromes constitute serious threats to human health as major risk factors for chronic diseases such as cardiovascular disease and type 2 diabetes mellitus [1,2].

Regulator of G-protein signaling (RGS) proteins are GTPase-activating proteins (GAPs) that, by associating with Gαi-subunits, negatively regulate G-protein-couples receptors (GPCR) signaling [3-6]. Some members of the RGS family proteins have been associated with obesity, inflammation, and impaired insulin sensitivity: RGS9 was shown to regulate body weight [7,8], RGS6 variants increased fat intake in Hispanics [9], RGS5 exaggerated obesity, inflammation, and insulin resistance after 24-wks of high-fat diet (HFD) feeding [10], and the 1114G allele of RGS2 gene was positively associated with obesity in a hypertensive population [11]. In addition, the glucose-dependent insulinotropic peptide-stimulated insulin secretion was attenuated by overexpression of RGS2 in βTC3 cells, suggesting a potential role of RGS protein in modulating desensitization of glucose-dependent insulinotropic peptide receptor, which is associated with impaired insulin secretion in type II diabetic patients [12]. Moreover, RGS16 has been implicated in the inflammation-induced activation of T lymphocyte [13], and overexpression of RGS16 was found to be a stimulator of glucose-induced insulin release in human and mouse islets and an inhibitor of somatostatin [14].

RGS10 is one of the smallest protein in RGS protein family [15]. RGS10 is abundantly expressed in brain, testis, lymph nodes, and bone marrow [16]. Arrays of studies have demonstrated a key role of RGS10 in the regulation of inflammation processes in the central nervous system

(CNS) and peripheral system. Microglia, the primary innate immune cells in the CNS, displayed dysregulated inflammation gene expression including elevated production of tumor necrosis factor (TNF), interleukin (IL)-1β, IL-6, IL-10, IL-12, and the chemokine (C-X-C motif) ligand in RGS10 knockout (KO) mice compared to microglia from wild-type (WT) mice [17]. RGS10 limits proinflammatory cytokines production in microglia by negatively regulating nuclear factor (NF)-κB pathway [18]. Also, KO macrophage exhibited increased TNF and IL-1β upon lipopolysaccharides (LPS) treatment [19], indicating RGS10 as a negative regulator of the inflammatory response. While emerging studies are focusing on neuro- and immunoprotective effects of RGS10, its role in the metabolic system has never been studied.

Green tea has been known for the protective activity against obesity [20,21], cardiovascular disorders [22,23], diabetes [24,25], and various types of cancers [26,27] through exerting its antioxidant and anti-inflammatory effects. Green tea attenuates HFD-induced obesity and inflammation characterized by reducing proinflammatory signaling molecules such as TNF [28-31]. Interestingly, (−) Epigallocatechin-3-gallate (EGCG), the major polyphenols found in green tea-induced the expression of RGS10 and RGS14 in human colon cancer cells [32], which implied RGS10 as a target gene for tea polyphenols.

Insulin resistance and metabolic syndromes are closely interrelated with chronic inflammation [33], and RGS10 plays an important role as a negative inflammatory mediator. Obesity and metabolic disorder induced by HFD in mice is a well-studied model of those conditions in human; therefore, we selected this model to test the following hypothesis: (1) that RGS10 KO would result in increased insulin resistance and inflammation, regardless of diet, but in particular, would exaggerate the effects of the HFD and (2) oral consumption of green tea extract would not reverse the metabolic effects of HFD in KO mice.

Materials and Methods

Animals and Study Design

KO mice (B6; 129S5-Rgs10Gt (IRESBetageo) 421Lex) were first generated by Lexicon Genetics by applying random-targeting technology [34] and re-derived in C57BL/6 strain and back-crossed for over 10 generations as previously described [17,18]. Five week old male C57BL/6J WT mice were purchased from Jackson Laboratory. KO (n=21) mice and WT mice (n=20) were randomly assigned to the following dietary groups for 8 weeks: low-fat diet (LFD, 10% kcal from fat), high-fat diet (HFD, 60% kcal from fat) or high-fat diet containing 2% green tea extract (wt:wt) (HFD+GTE). LFD and HFD were purchased from Research Diets, Inc (New Brunswick, NJ). Powdered green tea extract (GTE, 90M-B Sunphenon, Taiyo International, Inc. Minneapolis, MN) contains >75% total catechins including >40% EGCG and <10% Caffeine as verified by HPLC. The dose of GTE selected was based on that used in our previous studies [35,36]. The ingredient composition of the diets is shown in Table 1. Mice were housed in shoebox cages with free access to food and water in a pathogen-free, climate-controlled facility. Food intake and body weights were monitored every 3-4 days. After 7 wks on respective diets, glucose tolerance test and insulin resistance test were performed as previously described [37]. At the end of the 8-wks treatment period, after a 4 hrs fast mice were euthanized with anesthetic (vaporized isoflurane, 1 - 5%) followed by cervical dislocation. Blood was collected by cardiac puncture and tissues were harvested, weighed and snap-frozen in liquid nitrogen and stored at -80 ºC [38]. The study protocol was approved by the Institutional Animal Care and Use Committee at the University of Georgia.

Glucose Tolerance Test (GTT) and Insulin Resistance Test (IST)

GTT and IST were performed in succession (3 days apart) on the same animals. Mice were fasted for 3 hrs prior to the experiments. Following the fasting, glucose (2 g/kg BW, Sigma-Aldrich, St. Louis, MO, USA) was administered through oral gavage and blood glucose level was measured using a glucometer (TRUEresult®, Nipro Diagnostics, Fort Lauderdale, FL) by serial tail bleeds at various time points (0, 15, 30, 60, 90, and 120 min). Following 3 days of recovery, insulin (0.5 IU/kg, Sigma-Aldrich, St. Louis, MO, USA) was intraperitoneal injected after 3 hrs of fasting and blood glucose level was measured at various time points (0, 15, 30, 60, 75, 90, 105, and 120 min). During IST, those animals with blood glucose levels lower than 40 mg/dl for two consecutive time points were given a single bolus of glucose (20 mg/dl) to prevent them from developing severe signs of hypoglycemia [39]. HOMA-IR index was calculated to indicate insulin resistance as (fasting serum glucose (mg/dl) × fasting serum insulin (μg/ml)/22.5) [40].

Serum insulin and leptin

Serum was separated by centrifuging blood at 10000 × g for 5 min in 20 ℃ in Microvette®200 Z-Gel (SARSTEDT, Germany) immediately after the blood collection and stored at -80℃ until analysis. Serum insulin (Crystal Chem, Downers Grove, IL, USA) and leptin (Enzo Life Science Inc., Farmingdale, NY, USA) were measured by enzyme-linked immunosorbent assay (ELISA) in accordance with the manufacturer's instructions using a Molecular Devices M5 microplate reader.

Tissue homogenization and Quantitative real-time PCR

Liver and adipose tissues were isolated from the LFD fed WT and KO, HFD-fed WT and KO mice. The adipose tissues were homogenized using rotor-stator homogenizer isolation. Total RNAs were extracted using the AurumTM Total RNA Mini Kit (BioRad) following the

manufacturer's instructions. The RNAs were quantified using the NanoDrop and their integrities were further confirmed by locating the 18S and 28S bands on a 2 % agarose gel. One microgram of total RNA was isolated from cells using the AurumTM Total RNA Mini Kit (BioRad), treated with DNaseI, and reverse transcribed using Superscript II RNase H- reverse transcriptase (Invitrogen). QPCR was performed using SYBR Green in a 384-well format using a QuantStudio™ 6 Flex (Thermo Fisher Scientific). Oligonucleotide primers for QPCR were obtained from Integrated DNA Technologies (Coralville). Primer sequences for CD11b, CD68, TNF, IL-6, YM1, Fizz1, and RGS10 (available upon request) were validated and used for gene amplification. Levels of mRNA expression were normalized to those of the mouse house-keeping genes, actin, and GAPDH. Values represent the mean value of triplicate samples +/- SEM. Data are representative of at least two independent experiments. The relative quantification (RQ) value of each gene was compared to the gene expression of the LFD WT mice group.

Statistical analysis

Power analyses, outlined in the 3rd Edition of Design and Analysis, by Geoffrey Keppel [41] were used to estimate group sizes. The number of animals was based on our published work [35,36], and our studies and estimates per group for physiologic outcome measures yielded power values >0.80 with $\alpha = .05$. Data expressed as means ± S.E.M., were analyzed using Graph Pad Prism (Version 7.0; GraphPad Software, Inc.; San Diego, CA, USA). Student's *t*-test, one-way or two-way analysis of variance (ANOVA) with Tukey HSD (honestly significant difference) or Bonferroni post-hoc tests were applied to evaluate mean differences between groups.

Results

RGS10 deficiency exacerbates HFD-induced obesity in mice

We hypothesized that RGS10 plays a role in metabolic homeostasis based on our previous studies on its role in chronic inflammation and macrophage activation [19]. To determine the role of RGS10 on weight gain in response to dietary fat content, WT and KO mice were fed LFD or HFD. After 8 wks of LFD there was no difference in body weight between WT and KO mice (**Table 2, Figure 1A, and 1C**). However, HFD fed KO mice gained significantly more weight compared to the WT counterparts while the food intakes were not different between WT and KO mice (**Table 2, Figure 1B, and 1C**). Thus, KO mice are more susceptible to dietary fat-induced body weight gain.

For LFD-fed mice, there was no significant difference in tissue weights between WT and KO mice (**Table 2, and Figure 1D**). However, HFD-fed KO mice had significantly larger epididymal white adipose tissue (eWAT), retroperitoneal WAT (rpWAT) and liver tissue than WT counterparts (**Table 2, and Figure 1E**). We have also observed the larger subcutaneous adipose tissue depots in HFD-fed KO mice were larger in HFD-fed WT mice (data not shown). Body weight is highly correlated with rpWAT ($r=.802$, $p<.0001$) and liver mass ($r=.802$, $p<.0001$), suggesting that RGS10-related weight gain exacerbated by dietary fat may be associated with the increase in adiposity and liver mass.

RGS10 deficiency exaggerated glucose intolerance and insulin resistance.

Although body weight gain of WT and KO mice following LFD was not significantly different, KO mice fed LFD displayed impaired glucose tolerance following an oral glucose challenge which was not observed in WT counterparts. As shown in **Figure 2A** and 2**B**, blood glucose level of WT mice fed LFD returned back to the baseline level at 60-90 min following the

glucose challenge, whereas that of the mice fed HFD was back to the baseline level after 180 min.

KO mice fed HFD showed significantly higher levels of blood glucose than WT mice fed the same diet, indicating that RGS10 deficiency exaggerates glucose intolerance regardless dietary fat contents (**Figure 2B, and 2C**).

While KO mice displayed impaired glucose tolerance regardless of dietary fat contents, significant reduction of insulin sensitivity was observed only following HFD feeding (**Figure 2D, 2E, and 2F**). In WT mice, blood glucose level dropped and recovered by 120 min following intraperitoneal insulin challenge regardless the dietary fat contents. Similarly, KO mice fed LFD recovered the decreased blood glucose level to the baseline by 90 min post insulin challenge. However, KO mice fed HFD had a significantly elevated fasting glucose level and did not respond to insulin challenge in the same manner as that observed in other three groups (**Figure 2E-2H**). It appears that they had an exaggerated stress response to the injection procedure (15 min post-injection), and glucose levels rebounded to significantly higher levels after exogenous insulin administration, most likely in response to glucagon secretion and sympathetic activation [42].

To better access glucose tolerance and insulin sensitivity in these mice, we measured fasting serum insulin level and calculated homeostasis model assessment of insulin resistance (HOMA-IR) index (**Figure 2G, and 2H**). Following LFD, serum insulin levels and HOMA-IR index in KO mice were not significantly different compared to those in WT mice. However, HFD fed KO mice showed a higher level of fasting serum insulin ($p=.0002$) and HOMA-IR ($p=.0111$) compared to HFD fed WT mice.

Leptin is an adipocyte-derived hormone and involved in the regulation of energy homeostasis and glucose metabolism [43]. We observed higher serum leptin level in HFD-fed mice compared to LFD mice, both WT and KO (**Figure 2I**). In addition, KO mice had higher leptin levels than WT mice after both LFD and HFD, although the leptin levels were markedly increased in the KO HFD mice. Across all mice serum leptin level was positively associated with body weight (r=.913, *P*< .0001), liver mass (r=.788, *P*<.0001), and rpWAT mass (r= .933, *P*<.0001). Serum leptin level was also strongly correlated with serum insulin level (r=.931, *P*<.0001), supporting the interaction of these 2 hormones.

RGS10 deficiency augmented inflammation in the liver and adipose tissue during HFD-induced obesity.

Obesity and metabolic syndromes are often accompanied by abnormally increased inflammation and RGS10 functions as a negative inflammatory modulator [17-19]. Therefore, we measured the levels of inflammatory genes expression including CD11b, CD68, IL-1β, and TNF in the liver, which is the primary tissue that regulates glucose metabolism. Our data show that there are no significant differences in expression of inflammatory cytokine genes in either liver or adipose tissues of LFD-fed KO mice compared to LFD-fed WT mice (**Figure 3A-3D**). However, the expressions of CD11b, CD68, and IL-1β were significantly increased in HFD-fed KO mice compared to those in WT mice, indicating elevated inflammation in the liver of HFD-fed KO mice (Figure 3E-3H). The change in TNF gene expression in liver of HFD-fed KO vs WT mice did not reach significance.

Similarly, we measured the expression of inflammatory adipokine genes including IL-6 and TNF in adipose tissues. We found that expression of IL-6 in adipose tissue of HFD-fed KO mice was more than three-fold higher than that in HFD-fed WT mice. We also measured

macrophage alternative M2 activation markers including YM1 and Fizz1 in adipose tissues. The mRNA levels of both YM1 and Fizz1 were significantly lower in the adipose tissue of HFD-fed KO mice compared to those in HFD-fed WT mice while there were no differences between LFD-fed KO and WT mice (Figure 4).

The differential expressions of RGS10 in the liver and adipose tissue in HFD-fed WT mice

We explored whether dietary fat content influences RGS10 gene expression in the WT animals. The gene expression of RGS10 in the liver of WT mice fed HFD was significantly lower than mice fed LFD. Interestingly, the expression level of RGS10 in adipose tissue increased three-fold in HFD-fed mice than that of LFD-fed mice. This result shows that dietary fat content can modulate RGS10 gene expression in a tissue-specific manner that was up-regulated in adipose tissue while suppressed in the liver after long-term HFD (**Figure 5**).

Green tea extract ameliorates HFD-induced obesity, impaired glucose tolerance and insulin resistance in KO mice.

GTE has been shown to decrease body weight gain and insulin resistance induced by HFD [30,31,36,44,45]. To better understand the role of GTE in RGS10 deficiency, we measured body weight, glucose tolerance and insulin resistance following oral consumption of GTE along with HFD in RGS10 KO mice. As noted above, the response to GTE in WT mice has been demonstrated in several previous studies [30,31], so we did not include this group in our experiment. We tested GTE only in the KO mice fed HFD because that group had the most exaggerated responses and GTE has previously been shown to reduce weight gain and improve metabolic parameters in WT mice fed HFD. As shown in Figure 6A, GTE inhibited weight gain by 41% ($p<.0001$) in RGS10 KO mice fed HFD. In addition, GTE ameliorated impaired glucose tolerance and insulin resistance exaggerated by HFD in KO mice (**Figure 6B, and 6C**), thus

indicating that our original hypothesis that the anti-obesity and anti-diabetic function of GTE are associated with RGS10 was not accepted.

Discussion

In the current study, we demonstrated that (1) RGS10 plays an important role in glucose tolerance and leptin secretion, regardless of diet composition and body weight and adiposity, (2) in the absence of the RGS10 protein, the effects of a high fat diet on weight gain, glucose metabolism and inflammation are exacerbated; (3) green tea extract was effective in ameliorating the effects of RGS10 KO on body weight and glucose metabolism.

HFD induces insulin resistance and obesity in rodent animal models [46,47]. Consistent with previous studies, HFD induced impaired glucose tolerance and increased HOMA-IR index in the present study. Both serum leptin and insulin level were increased, which is in agreement with human studies reporting that these hormones were elevated in obese subjects [48-50]. Insulin and leptin are closely associated with inflammation and reciprocally regulate one another in the progress of diet-induced obesity [51,52]. Insulin regulates leptin production *in vivo* and *in vitro* [53,54] while leptin inhibits insulin binding ability and impairs insulin activity in adipocytes [55-57]. Hyperleptinaemia is often observed in obese type 2 diabetes [48-50], indicating these subjects might be resistant to leptin signal [46], but the mechanism of responsible for leptin resistance in the obese and diabetic subjects has yet to be fully elucidated [58].

In our study, KO mice displayed increased circulating leptin levels and glucose intolerance that were exacerbated by HFD. In the HFD-fed KO mice, the high leptin level was associated with insulin resistance. Moreover, leptin regulates macrophage activation [59] whereas RGS10 acts as a negative regulator of the inflammatory response [19], suggesting that RGS10 gene

product may act, at least in part, through leptin in the development of insulin resistance and inflammatory responses induced by HFD.

As we demonstrated previously, RGS10 KO mice displayed dysregulated inflammatory gene expression, increased production of inflammatory cytokines including TNF, IL-1, IL-6, IL-10, IL-12, and the chemokine CXCL1, and enhanced NF-κB pathway activation in microglia [17,18]. Obesity-induced inflammation has been identified as a key component in the pathogenesis of insulin resistance and type 2 diabetes [60]. As shown previously, excessive adiposity induces adipocyte dysfunction, increases the infiltration of immune cells into adipocytes, and leads to abnormal production of adipokines, including IL-6 and TNF which mediate the inflammatory response in adipose tissue [61]. CD11b was previously shown to regulate diet-induced abnormal adiposity and insulin resistance [62]. We found that KO mice fed HFD had a sustained inflammation as indicated by increased expressions of CD11b and CD68 in the liver, thus implicating an augmented inflammatory response associated with absence of RGS10 expression [62]. YM1 and Fizz1 are representative markers for alternatively activated (M2) macrophages, which are up-regulated in response to IL-4 [63,64]. Macrophages from microglia of RGS10 KO mice displayed increased expression of proinflammatory cytokines including IL-1β and TNF upon lipopolysaccharide treatment and decreased YM1 and Fizz1 expression following IL-4 administration [18 19]. In adipose tissue of HFD-fed KO mice, an inflammatory M1 phenotype characterized by increased expression of IL-6 was found while the expression of M2 activation markers YM1 and Fizz1 was increased, which suggests that the metabolic disorders in KO mice may be mediated by RGS10's role in immune responses.

RGS10 gene expression is altered by physiological and pathological processes such as inflammation, aging, and cancer [65-67]. RGS10 gene expression was suppressed by DNA

methylation and histone de-acetylation in ovarian cancer cells [65]. RGS10 expressions in immune cells was largely influenced by age, with an increased expression in B cells, monocytes, and granulocytes, and decreased expression in microglia with aging [66,67]. Here, for the first time, we report dietary fat content as a potential regulator of RGS10 expression in the liver and adipose tissue. It is important to note that in the present study, HFD modulated RGS10 expression in a tissue-dependent manner, and this may account for the inflammatory response we observed in these tissues. It is possible that the differential effect of HFD on RGS10 expression in these tissues might result in different patterns of expression or infiltration of immune cells. Further in-depth studies need to be followed to investigate whether the protective effect of RGS10 on the development of chronic diseases is through its role in anti-inflammatory mechanisms or its novel function in metabolic homeostasis and how these might be interrelated.

Green tea and its catechins have been shown to alleviate weight gain, adiposity, hyperglycemia, and insulin resistance [30,31] by attenuating inflammation and oxidative stress in HFD-induced obese mice [68]. EGCG has also been shown to increase the levels of RGS10 expression in human cancer cells [32], suggesting that the anti-inflammatory action of green tea and its catechins may be mediated by through modification of RGS10 expression. However, in the present study, GTE was effective in ameliorating weight gain, impaired glucose tolerance and insulin resistance in the HFD-fed RGS10 KO mice, suggesting that the protective effect of GTE on the development of metabolic disorder induced by HFD may not be directly related to an effect on RGS10 expression in liver or adipose tissue.

Conclusions

In summary, our findings suggested the role of RGS10 in protecting against obesity and maintaining glucose metabolism and insulin sensitivity, in addition to its role in the anti-

inflammatory response. This is significant since RGS10 may serve as a novel therapeutic target for metabolic disorders modulating both inflammation and metabolic homeostasis.

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Table 4.1. Ingredient composition of the diet (g/kg)[1]

Ingredient	LFD	HFD	HFD+GTE
Casein	189.6	258.0	254
L-Cysteine	2.8	3.9	3.8
Corn starch	479.8	0	0
Maltodextrin	118.5	161.3	158.8
Sucrose	65.2	88.8	87.4
Cellulose	47.4	64.5	63.5
Soybean oil	23.7	32.3	31.8
Lard	19	316.1	311.2
Mineral mix S10026	9.5	12.9	12.7
DiCalcium Phosphate	12.3	16.8	16.5
Calcium carbonate	5.2	7.1	7.0
Potassium citrate, 1 H2O	15.6	21.3	21.0
GTE	0	0	20.0
Vitamin mix V10001	9.5	12.9	12.7
Choline bitartrate	1.9	2.6	2.5
Total	1000	1000	1000

[1] Six-wks old RGS10-KO and C57 Bl/6J wild-type mice were fed low-fat diets (LFD, 10% kcal from fat), high-fat diets (HFD, 60% kcal from fat) or high-fat diet containing 2% GTE (HFD+GTE) for 8 weeks.

Table 4.2. Body composition and food intake in WT and KO mice fed LFD and HFD[1]

	WT LF	WT HF	KO LF	KO HF	ANOVA
Initial body weight	20.12 ± 1.01^{a}	20.48 ± 1.26^{a}	15.80 ± 1.79^{b}	22.31 ± 2.76^{a}	<.05
Final body weight	25.91 ± 0.96^{ac}	36.81 ± 2.34^{b}	24.10 ± 3.03^{c}	44.89 ± 1.99^{d}	<.05
Food intake (g/day)	2.63 ± 0.28^{a}	3.36 ± 1.26^{b}	2.45 ± 0.27^{a}	3.03 ± 0.90^{ab}	<.05
Adipose mass[2]	0.50 ± 0.08^{a}	3.01 ± 0.43^{bc}	0.69 ± 0.28^{a}	3.29 ± 0.47^{c}	<.05
Brain mass	0.45 ± 0.02^{a}	0.45 ± 0.02^{a}	0.42 ± 0.03^{a}	0.43 ± 0.01^{a}	<.05
Heart mass	0.16 ± 0.03^{a}	0.19 ± 0.04^{a}	0.17 ± 0.04^{a}	0.19 ± 0.02^{a}	<.05
Liver Mass	1.02 ± 0.23^{a}	1.18 ± 0.12^{a}	1.03 ± 0.21^{a}	1.99 ± 0.50^{b}	<.05
Pancreas mass	0.10 ± 0.02^{a}	0.15 ± 0.08^{a}	0.11 ± 0.03^{a}	0.12 ± 0.03^{a}	<.05
Spleen mass	0.09 ± 0.07^{a}	0.10 ± 0.04^{a}	0.09 ± 0.02^{a}	0.10 ± 0.04^{a}	<.05

[1] Unit used are g if without specification. Means ± S.E. Values in a row not sharing a common letter differ (P<.05).

[2] Sum of epididymal and retroperitoneal white adipose tissue weight.

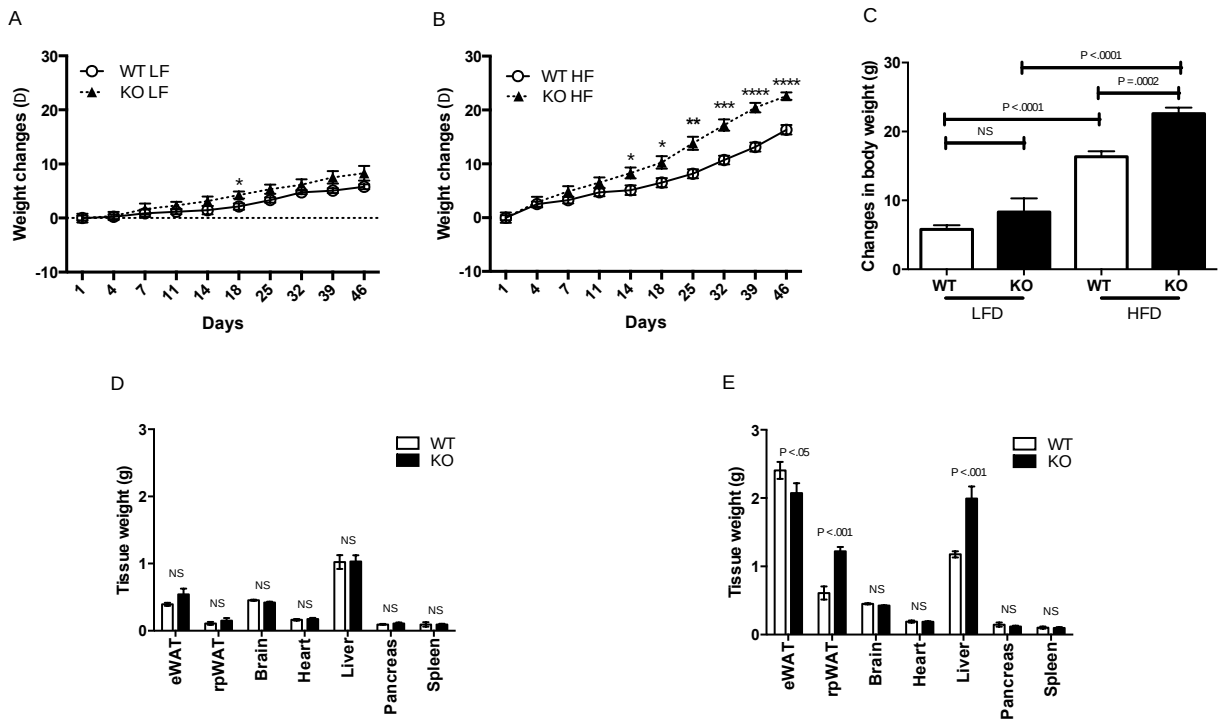

Figure 4.1. HFD-fed KO mice gained higher body weight and displayed increased adiposity. Body weight (A-C) and tissue weight (D-F) were measured following LFD (A and D) or HFD (B and E) for 8 wks. Data represents mean ± SEM; n= 5-8 mice per group. * $P < .05$, ** $P < .01$, *** $P < .001$, **** $P < .0001$, NS: not significant. LFD: low-fat diet; HFD: high-fat diet; eWAT: epididymal white adipose tissue; rpWAT: retroperitoneal white adipose tissue.

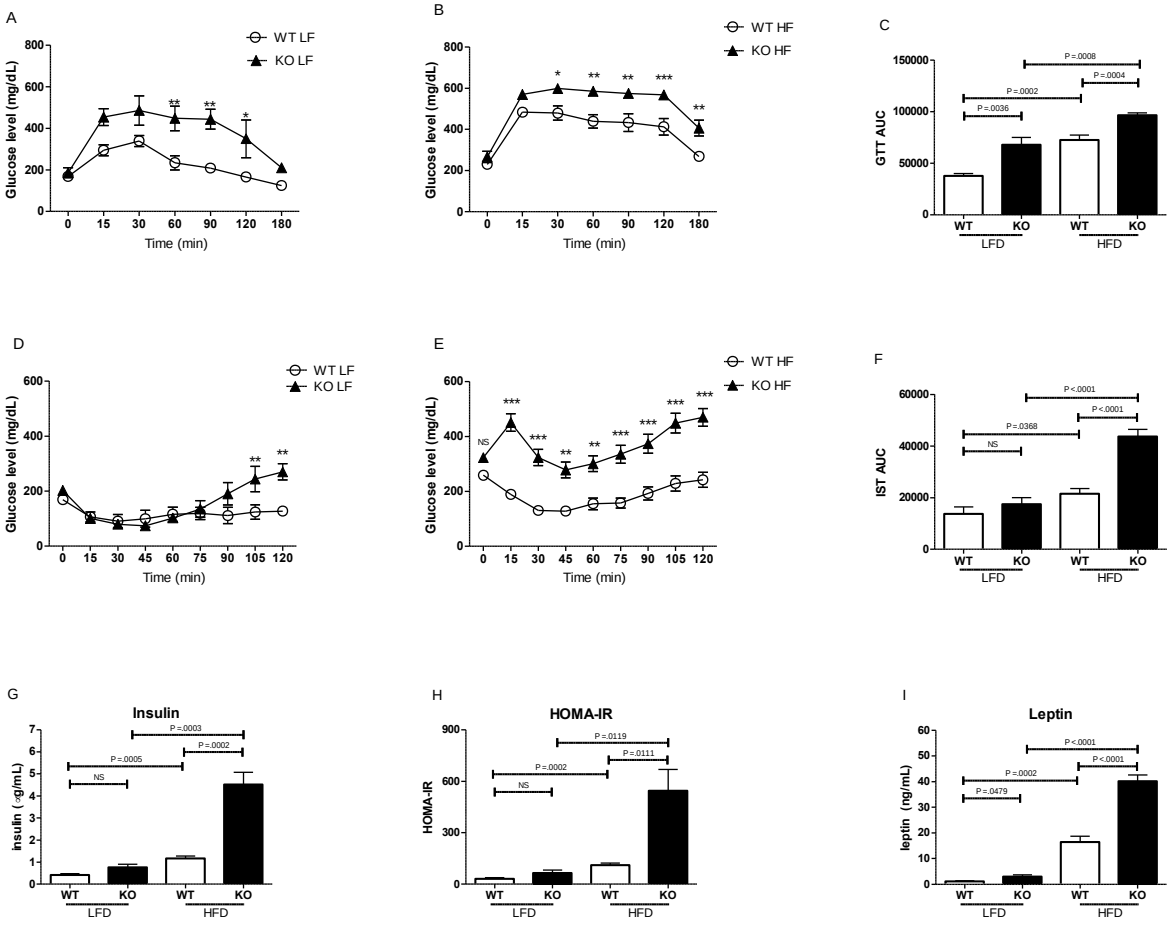

Figure 4.2. HFD-fed KO mice developed glucose intolerance and insulin resistance. Oral glucose tolerance test (GTT, A-C) and insulin sensitivity test (IST, D-F) were conducted following LFD (A and D) or HFD (B and E) for 7 wks. Serum insulin level (G), HOMA-IR (H) and serum leptin level (I) were measured. Data represent mean ± SEM; n= 5-8 mice per group. * $P < .05$, ** $P < .01$, *** $P < .001$, **** $P < .0001$, NS: not significant. LFD: low-fat diet; HFD: high-fat diet.

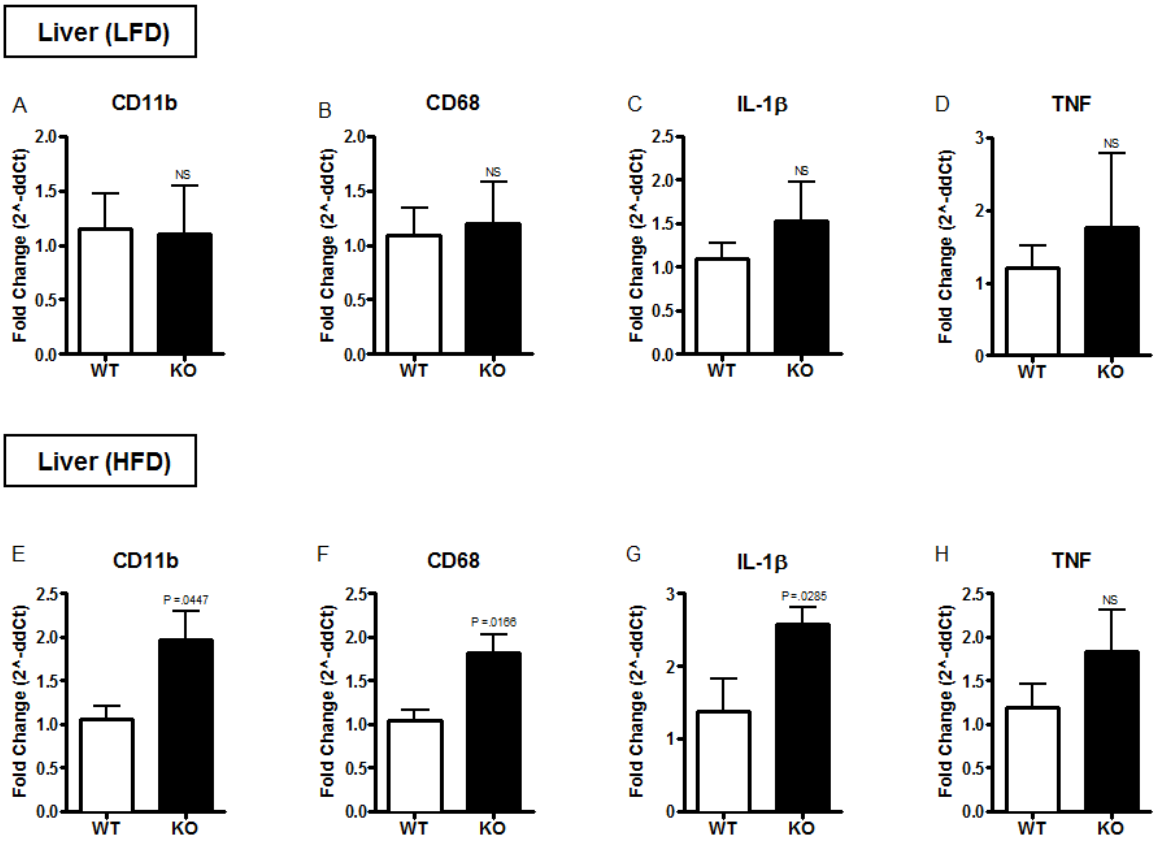

Figure 4.3. HFD-fed KO mice displayed higher inflammation in the livers. The expression of inflammatory markers including CD11b, CD68, IL-1, and TNF were measured by QPCR analysis in the livers of WT and KO mice fed LFD (A-D) or HFD (E-H) for 8 wks. Data represent mean of the fold changes of genes ± SEM; n= 5-8 mice per group. NS: not significant. LFD: low-fat diet; HFD: high-fat diet.

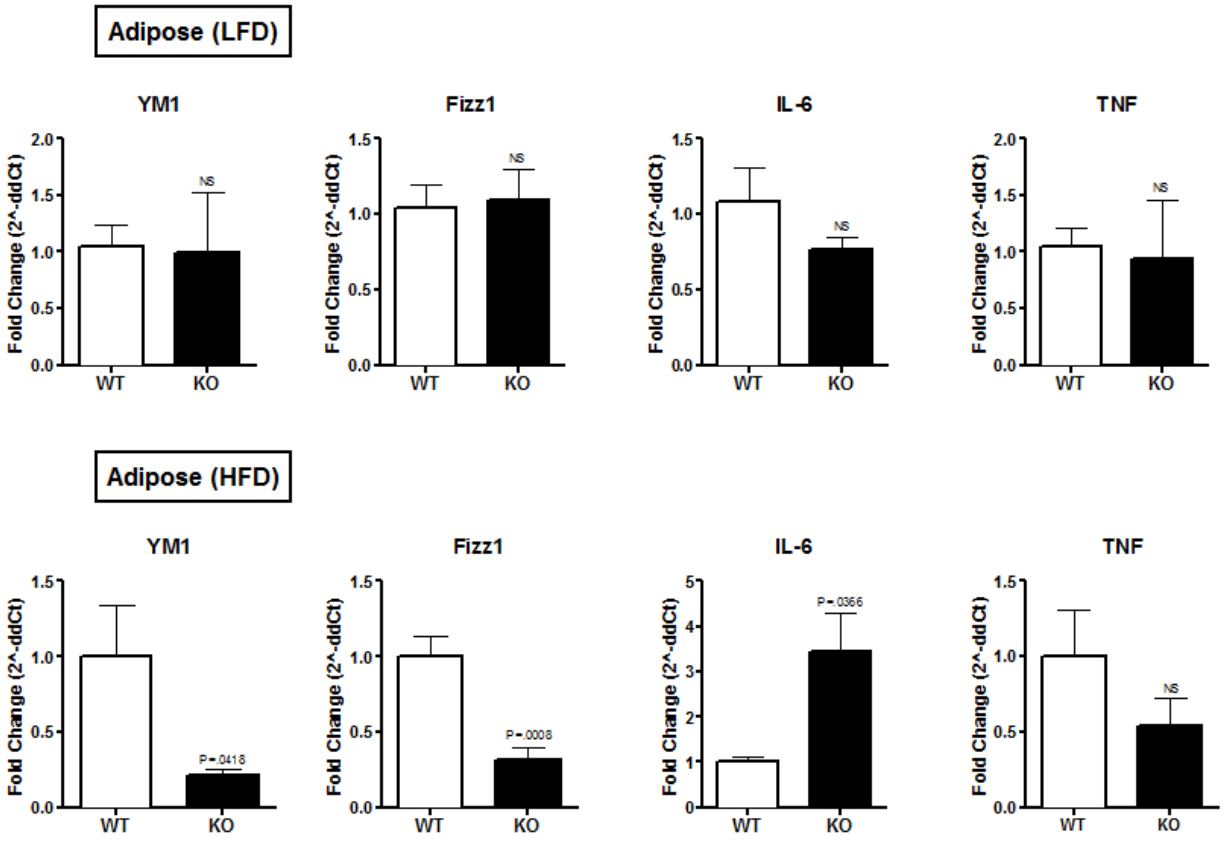

Figure 4.4. HFD-fed KO mice displayed lower anti-inflammatory M2 markers but higher proinflammatory M1 in the adipose tissue. The mRNA levels of IL-6, TNF, YM1, and Fizz1 in the adipose tissues from mice fed LFD (A-D) or HFD (E-H) for 8 wks. Data represent mean of the fold changes of genes ± SEM; n= 5-8 mice per group. NS: not significant. LFD: low-fat diet; HFD: high-fat diet.

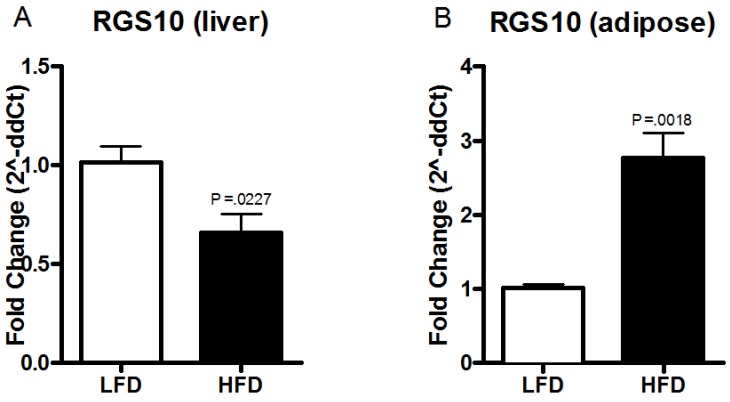

Figure 4.5. RGS10 gene expressions were differentially regulated in different tissues. Transcriptional expressions of RGS10 were examined in the liver (A), and adipose tissue (B) of WT mice fed LFD or HFD for 8 weeks. The fold changes of gene ± SEM; n= 5-8 mice per group. LFD: low-fat diet; HFD: high-fat diet.

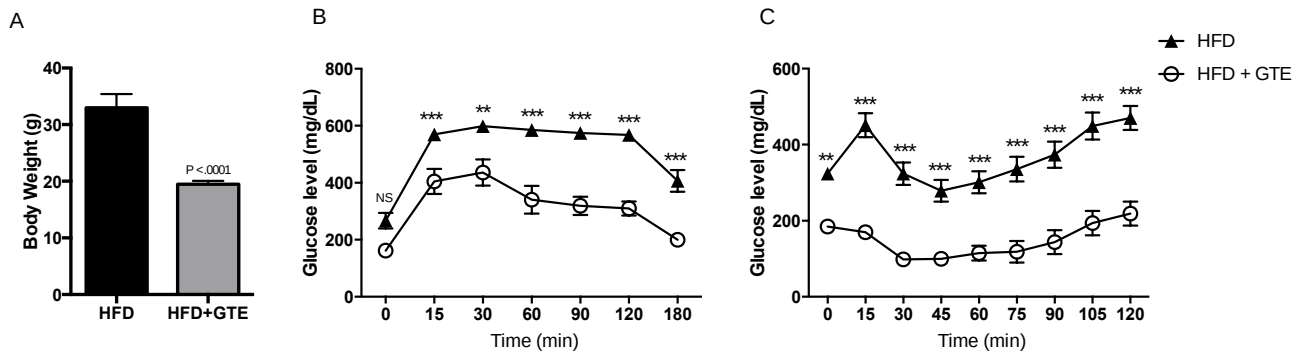

Figure 4.6. GTE ameliorated HFD-induced weight gain, impaired glucose tolerance and insulin resistance in KO mice. Body mass (A), glucose tolerance test (B), and insulin resistance test (C) were measured in KO mice after 8 wks feeding of HFD or HFD+GTE. Data represent mean ± SEM; n= 8 mice per group. * $P < .05$, ** $P < .01$, *** $P < .001$. LFD: low-fat diet; HFD: high-fat diet; HFD+GTE: high-fat diet containing 2% green tea extract (wt:wt).

CHAPTER 5[1]

EFFECT OF ACUTE INGESTION OF GREEN TEA EXTRACT AND LEMON JUICE ON OXIDATIVE STRESS AND LIPID PROFILE IN PIGS FED A HIGH-FAT DIET

1. Fang X, Azain M, Crowe-White K, et al. Effect of Acute Ingestion of Green Tea Extract and Lemon Juice on Oxidative Stress and Lipid Profile in Pigs Fed a High-Fat Diet. *Antioxidants (Basel).* 2019;8(6). Reprinted here with permission of the publisher.

Abstract

Green tea and its catechins have been shown to ameliorate high fat diet-induced oxidative stress and hyperlipidemia. However, low bioavailability of catechins limits their therapeutic potential. Lemon juice (LJ) has been suggested to enhance the bioavailability of catechins in vitro. This study investigated the antioxidative and hypolipidemic efficacy of a single dose of green tea extract (GTE) or GTE plus LJ (GTE+LJ) in high-fat diet fed pigs. Sixteen pigs ingested a single dose of GTE (190 mg/kg/day) or GTE+LJ (0.75 ml/kg/day) mixed with low fat (LF; 5% fat) or high fat (HF; 22% fat) diets and blood samples were collected for 24 hrs. Plasma catechin level peaked at 2 hrs, and gradually returned to baseline after 6 hours following the intake. The addition of LJ significantly increased plasma catechin level. The diet containing GTE did not lower plasma cholesterol and triacylglycerol (TG) concentrations, superoxide dismutase (SOD) and catalase activity, or malondialdehyde concentration in 24 hrs in HF-fed pigs. Addition of a single dose of LJ however significantly decreased plasma TG level in LF groups but did not cause further changes on any other markers compared to the GTE alone. Our findings indicate limited effect of a single meal containing GTE on plasma antioxidant enzymes, lipid profile, and lipid peroxidation in pigs and no significant synergistic/additive action of adding LJ to GTE within 24 hrs in pigs. A study with a longer treatment period is warranted to further understand the potential role of GTE in reducing HF diet-induced oxidative stress and the possible synergistic role of LJ.

Keywords: antioxidant enzymes; catechins; cholesterol; citrus fruits; lipid peroxidation; triacylglycerol

Introduction

Oxidative stress is a key component in numerous metabolic disorders and disease pathologies, such as diabetes, cardiovascular disease, and cancer [1]. Diet pattern is of great importance as a modulatory risk factor for oxidative stress in the body [2]. A high-fat (HF) diet often results in adverse metabolic outcomes where oxidative stress is increased by free radical production and an elevated inflammatory response characterized by macrophage migration and higher levels of inflammatory cytokines [3,4].

Green tea (*Camellia sinensis*) is a popular beverage consumed worldwide for its long history and numerous health benefits. The major polyphenolic compounds in green tea are (-)-epigallocatechin gallate (EGCG), (−)-epicatechin-3-gallate (ECG), (−)-epigallocatechin (EGC), and (−)-epicatechin (EC), among which EGCG is the most abundant, accounting for more than 50% of catechin [5]. Green tea and its catechins exhibit anti-oxidative and anti-inflammatory properties making them potentially potent therapeutics for diseases where these processes are critical negative elements [6-10]. Green tea supplementation has been shown to have beneficial effects by reducing oxidative stress, high blood pressure, lipid absorption and glucose intolerance induced by a HF diet.

However, findings with short-term supplementation are inconsistent. In humans, green tea supplementation for 7 and 28 days decreased systolic blood pressure in obese and prehypertensive women [11] and attenuated postprandial blood glucose and insulin response in overweight men [12], respectively. Walkowiak et al., [13] also reported a decrease in lipid digestion and absorption after a single dose of green tea extract in healthy human subjects. Inconsistently, two days of green tea intervention showed no beneficial effect on glucose, insulin, and free fatty acids levels [14] and on resting metabolism [15] in healthy men and women. In addition, a single dose

of green tea polyphenols did not ameliorate oxidative stress and muscle damage induced by exercise in healthy athletes [16]. These discrepancies suggested that one potential factor leading to these divergent outcomes may be related to the treatment period and emphasized necessity of elucidating acute single dose ingestion of catechins in the antioxidative efficacy of green tea.

Furthermore, the oral bioavailability of green tea catechins is very poor with most catechins being cleared through the gastrointestinal (GI) tract before making it into circulation [17]. An enhancement in uptake and delivery of catechins would significantly improve the beneficial efficacy of green tea. Green et al., [18] demonstrated that the addition of citrus juice modulates *in vitro* digestive recovery of green tea catechins and lemon juice showed the maximum catechin recovery among various citrus fruits. An *in vivo* study [19] suggested that ascorbic acid improves catechin's bioavailability in rats. Intriguingly, in humans, a greater consumption of citrus fruits was associated with a lower incidence of stroke in Japan [20] and a lower risk of fatal stroke in the UK [21]. Lemon has been reported to possess health-promoting activity related to cancer, cardiovascular diseases, obesity, gastrointestinal diseases, diabetes, urinary diseases, psychiatric diseases, and bone protection [22]. Lemon juice contains antioxidants such as ascorbic acid, phenols, and flavonoids and possesses antioxidant capacity [22,23]. In our current study, we hypothesize that the combination of green tea extract and lemon juice may have a synergistic effect on antioxidant activity due to the increase in catechin's bioavailability.

Therefore, the present study aimed to investigate the antioxidative efficacy of a single dose of green tea extract (GTE), and the synergistic effect of green tea and lemon juice (GTE+LJ) in combination with low-fat (LF) or HF diet in a pig model. This animal model has proven to be superior to rodent models in studying absorption and metabolism due to similarities in gastrointestinal structures and disease progression between pigs and humans [24,25].

Materials and Methods

Animals and Study Design

The cross-bred commercial line of healthy male and female pigs (n=16) were obtained from the University of Georgia swine farm at the age of 15 weeks with a body weight of 37-49 kg. After two weeks of acclimation in a temperature controlled facility, they were trained to consume a meal within 30 min for one week. A catheter was surgically implanted into the jugular vein of each pig and a 2-day recovery period was allowed prior to the experiment. Pigs were randomly assigned to four treatment groups: low-fat diet with green tea extract (LF+GTE) (n=5), high-fat diet with green tea (HF+GTE) (n=4), low-fat diet with green tea extract and lemon juice (LF+GTE+LJ) (n=3), and high-fat diet with green tea extract and lemon juice (HF+GTE+LJ) (n=4). Blood samples were collected before treatment as a baseline. Each animal at baseline measurement served as its own control. Blood samples were obtained through the jugular catheter at baseline (pretreatment), 1hr, 2hr, 3hr, 4hr, 6hr, 12hr, and 24hr post-consumption of experimental diets (Figure 1). This study was conducted in accordance with the University of Georgia Institutional Animal Care and Use Committee guidelines (project code: A2016 06-011-Y3-A5).

Treatments

All animals were maintained on a grower diet primarily composed of corn with a total of 3400 kcal/kg to meet normal metabolism and growth requirement according to NRC, 2012 [26]. Pigs in the LF group consumed the baseline diet throughout the study (5% fat diet). The HF diet was prepared by mixing the base diet with 20% lard (wt/wt) (designated as the 22% fat diet). The dose of GTE was selected based on literature reporting beneficial health effects in human subjects [27-29]. In order to accurately translate data from pigs to humans, the doses of GTE were

adjusted using standard FDA conversions between animal and human equivalent dose [30], which resulted in 1.3g/pig/day of GTE (90M-B Sunphenon, The Food Grade, non GMO powdered GTE, Taiyo International, Inc.), containing >75% (wt/wt) total catechins including >40% and <10% Caffeine EGCG as verified by HPLC. This is equivalent to 5 cups/day of human consumption which has been shown to decrease cardiovascular disease mortality in the Japanese population [31]. The dose of lemon juice (ReaLemon, Dr. Pepper Snapple Group) was selected based on the FDA reports of lemon juices used as ingredients [32]. The LF or HF diet were mixed with GTE (190mg/kg/treatment) or GTE+LJ (190mg/kg/treatment GTE+ 0.75ml/kg lemon juice/treatment).

Plasma Catechin Analysis

Blood was collected in EDTA-coated tubes, and plasma was separated by centrifugating blood at 2000 ×g for 10 min at 20 ℃. Plasma EGCG and EGC levels were analyzed using a previously validated ultra-high performance liquid chromatography (UPLC) method for analysis of tea catechins [33]. The Acquity UPLC system was interfaced with a photodiode array detector and a quaternary solvent manager (Waters Corporation, Milford, MA) and the instrument was fitted with an Acquity UPLC HSS T3 column (100mm x 2.1 mm, 1.8um) protected with a 0.2um in-line filter. Reference standards included EGCG (97%) and EGC (95%) purchased from Sigma (St. Louis, MO). Standard stock solutions were prepared in 3% acetonitrile and further diluted with 3% acetonitrile to obtain different concentration levels for preparation of standard curves.

Plasma Cholesterol and Triglyceride

To determine the effect of acute HF diet w/o GT and LJ feeding on the circulating lipid level. Plasma cholesterol and triglycerides (TG) were measured using colorimetric and

enzymatic standard methods according to the manufacturer's instructions (Fisher Diagnostics, Middletown, VA, USA).

Liver Function Tests

To determine whether the dose we chose causes any hepatotoxicity, liver alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, and gamma-glutamyl transferase (GGT) were measured using a clinical chemistry analyzer.

Plasma Lipid Peroxidation

With the aim of exploring the potential protective effect of GTE and GTE+LJ on oxidative stress, the plasma level of malondialdehyde was analyzed spectrophotometrically as an indicator of lipid peroxidation using thiobarbituric acid reactive substances (TBARS) assay kit (Cayman, Ann Arbor, MI, USA) according to the manufacturer's instruction.

Activities of Plasma Antioxidant Enzymes

To measure antioxidant defense after acute administration of test diets, plasma superoxide dismutase (SOD) and catalase enzyme activities were analyzed colorimetrically using superoxide dismutase assay kit (Cayman, Ann Arbor, MI, USA) and catalase assay kit (Cayman, Ann Arbor, MI, USA) according to the manufacturer's instructions.

Statistical Analysis

Data expressed as mean ± S.E. were analyzed using Graph Pad Prism (Version 7.00; GraphPad Software, Inc.; San Diego, CA, USA). One-way analysis of variance (ANOVA) with Tukey HSD (Honestly Significant Difference) post-hoc test and paired t-test were applied to evaluate mean differences between groups and between time points, respectively.

Results

Plasma Catechin Level

In order to determine the level of catechin in the systemic circulation, plasma EGCG and EGC were measured at 2, 4, and 6 hrs after the acute intake of LF diet with GTE or GTE+LJ in pigs. In both GTE and GTE+LJ groups, plasma EGCG and EGC concentration increased dramatically upon ingestion and peaked at 2 hrs after the intake (EGCG: 49.77±6.26, 84.66±8.97 nmol/L; EGC: 25.28±3.07, 38.36±8.65 nmol/L, EGCG+EGC: 75.05±6.20, 123.01±32.59 nmol/L in GTE and GTE+LJ, respectively). The peak concentration of EGCG in GTE+LJ group was 1.7-fold higher than GTE group (p=0.03). Similarly, the peak circulating levels of EGCG+EGC in GTE+LJ was 1.64-fold higher than GTE group (p=0.06). Plasma catechin concentration went back to baseline (below 2 nmol/L) 6 hrs following the treatment in both GTE and GTE+LJ groups (Figure 2).

Plasma Lipid Profile

Green tea and its catechins have been documented for their hypolipidemic activity. Plasma cholesterol and TG were measured after a single-dose of GTE or GTE+LJ with LF or HF diet in pigs. In our study, different fat content did not alter postprandial cholesterol and TG levels comparing LF+GTE and HF+GTE groups. In LF diet-fed groups, postprandial cholesterol level decreased up to 7% at 4 hr which then returned to baseline, while a combination of GTE+LJ did not further influence plasma cholesterol within 24 hrs (Table 1 and Figure 3). In HF diet-fed groups, cholesterol was not altered by acute intake of GTE and combination of GTE+LJ.

In LF+GTE group, plasma TG concentration was decreased up to 20% at 1 hr and 6 hr timepoint compared to preprandial level which returned to baseline levels within 24 hrs (Table 2). In LF+GTE+LJ group, plasma TG concentration dropped up to 53% 2-12 hrs following the intake compared to preprandial level (Table 2). Plasma incremental TG AUC was significantly lower in LF+GTE+LJ group compared to LF+GTE group (p=0.01) (Figure 4). In HF fed-groups,

postprandial TG concentration significantly decreased at 4, 6, 12 hrs and 3, 4, 6 hrs compared to baseline level in HF+GTE group and HF+GTE+LJ groups, respectively (Table 2). However, between HF+GTE and HF+GTE +LJ groups, there was no significant difference in the TG concentration.

Liver Function Tests

The doses of GTE and LJ tested in the current study did not cause liver toxicity. No significant changes were noticed in ALT activity, AST activity, total bilirubin, and GGT levels (data not shown).

Plasma Lipid Peroxidation

HF diet has been reported to induce an elevation in plasma lipid content and potentiate systemic oxidative stress [34,35], while chronic treatment with catechins has been reported to normalize elevated lipid peroxidation induced by HF diet in rats [36]. Thus, we measured plasma malondialdehyde (MDA) concentration to investigate whether acute ingestion of GTE or the combination of GTE+LJ would be sufficient to reduce lipid peroxidation in HF-fed pigs. As shown in Table 3 and Figure 5, a single dose of HF diet had no significant effect on postprandial plasma MDA level comparing LF+GTE and HF+GTE groups. In LF diet-fed groups, MDA concentration did not change following GTE or GTE+LJ ingestion. In HF diet-fed groups, MDA level tended to decrease at 1 and 3 hrs by 16% postprandial in HF+GTE group, which went back to baseline after 4 hrs. Addition of LJ with GTE also decreased MDA up to 45% within the first 2 hrs of ingestion but then recovered to baseline level after 4 hrs (Table 3).

Activities of Plasma Antioxidants Enzymes

Activities of plasma SOD and catalase were measured to indicate antioxidant defense in the HF diet-fed pigs after acute exposure to either GTE or GTE+LJ. As shown in Table 4 and

Figure 6, different fat content did not exert a significant effect on postprandial plasma SOD activity comparing LF+GTE and HF+GTE groups. However, Plasma incremental SOD AUC showed a trend of decrease in HF+GTE+LJ compared to LF+GTE+LJ group. In LF+GTE group, the plasma SOD activity increased 68% from 1 hr following the meal, returned to the baseline level at 6 hrs, and again increased up to 139% at 24 hrs following intake comparing to preprandial level. The GTE+LJ group showed a similar trend as GTE alone but no significance was observed which may be due to the small number of animals in the group. In HF diet-fed pigs, no change was observed in the activity of SOD. The addition of LJ did not exert an apparent effect on plasma SOD activity comparing HF+GTE and HF+GTE+LJ groups (Table 4 and Figure 6). As shown in Table 5 and Figure 7, when GTE was consumed with HF diet, postprandial catalase activities tended to be lower compared to LF+GTE group. In LF diet-fed pigs, postprandial plasma catalase activities tended to be higher regardless of fat content comparing LF+GTE+LJ to LF+GTE and HF+GTE+LJ to HF+GTE groups, however, none of these reached the statistical significance cut-off point. Taken together, our data revealed that GTE had a limited influence on plasma SOD and catalase activity, and LJ did not modulate this effect within 24 hrs.

Correlation between Plasma Antioxidant Enzymes and Triglycerides

Pearson correlation analysis revealed a positive correlation between plasma catalase activity and plasma SOD activity (r=0.26, P=0.01), and a negative correlation between plasma catalase activity and plasma TG level (r=-0.21, P=0.03) (Figure 8).

Discussion

Green tea and its catechins possess therapeutic potential for chronic diseases by mitigating oxidative stress and hyperlipidemia. The low bioavailability of catechins limit their

therapeutic potential, and there are numerous studies attempting to overcome this challenge by enhancing their bioavailability including strategies such as encapsulation, nanoparticle delivery or combining with other foods. One of the safest and most novel approaches is the combination of citrus fruits with green tea, which was been found to increase the bioavailability of green tea catechin in an in vitro digestion model [18]. In accordance with findings reported by Huang Y.B. et al., and Lee M, et al., we found plasma concentration of catechin peaked at 2 hrs upon single dose ingestion and gradually decreased to baseline between 6-8 hrs after oral consumption both in rodents and humans [37,38]. Moreover, the present study reported a significant increase in circulating catechin level when GTE was ingested in combination with LJ, which is in agreement with Peters C.M, et al.,'s finding that the addition of ascorbic acid and sucrose significantly increased plasma EGCG and EGC concentration within 2 hrs after the ingestion in rats [19]. We hypothesize that several factors might have contributed to the observed improvement in blood catechin levels with GTE+LJ compared to LJ alone. Polyphenol rich tea drinks are susceptible to form precipitates refered to as tea cream, caused by the interations of catechin, proteins, and other polyphenols [39]. The precipitation is formed more readily under acidic conditions like gastric environment [40]. Galated catechins including EGCG, GCG, ECG, and CG were found to have higher tendency to form cream compared to other catechins [41]. The ascorbic acid in LJ was shown to improve the circulating catecin level likely by improving the stabilization of these labile catechins in the gastrointestinal tract [18]. Additionally, ascorbic acid was found to increase the stability of catechin and its derivatives especially for EGC and EGCG in vitro [42], suggesting a possible explanation for the increased plasma catechin levels in GTE+LJ group.

In accordance with previous research, postprandial cholesterol did not change significantly after the intervention meal [43,44]. In addition, in this study, we did not observe a

significant difference in plasma TG levels between LF+GTE and HF+GTE groups, suggesting that the one-time increase in fat content did not impact plasma TG when HF was consumed with GTE. This is likely due to the hypolipidemic effect of green tea. In normal human subjects, postprandial plasma TG increases in response to a HF meal around 2 hrs [45]. In pigs, postprandial TG decreased 1 hr after the meal and then increased after 2hrs [46]. GTE significantly decreased plasma and hepatic TG 6 weeks after feeding in mice [47]. Another study found that 4-day EGCG supplementation decreased liver and plasma TG level compared to the HF diet-fed controls in mice [48]. Additionally, green tea catechin has also been reported to decrease postprandial plasma TG in human subjects [49]. In this study, postprandial TG levels of all treatment groups were decreased within 6 hrs after the meal, which was further decreased by the addition of LJ under normal conditions in a pig model. These findings may suggest that GTE, especially when consumed with LJ, prevented the increase of plasma TG level after a meal.

We found that when combined with GTE, higher dietary fat content resulted in decreased plasma MDA concentration in the HF+GTE group compared to the LF+GTE group. Interestingly, previous findings report that a chronic or repeated single HF diet increases the plasma MDA concentration [50,51]. Multiple factors can be attributed to different biological reactions to a HF meal including, macronutrient composition, different types of fat, lifestyles, and bioactive food compounds within a food matrix. Montes-Nieto R. et al.. found that postprandial MDA increased significantly compared to the preprandial level after ingestion of glucose; however, this was not seen when ingesting a lipid diet wherein the postprandial circulating TBARS remained unchanged throughout the trial compared to baseline level following the ingestion of poly-unsaturated triglycerides nutrition supplement in young adults [52]. In another study that investigated the postprandial antioxidant defenses in physically active and

inactive men, TBARS in physically active men showed a decreasing trend compared to baseline level after a HF meal [53]. In this study, the decrease of MDA concentration observed in HF+GTE diet compared to LF+GTE group may be a compensating effect due to the activation of the antioxidant effect of GTE when combined with HF diet, suggesting GTE alone, or the combination of GTE and LJ was able to suppress the elevation of plasma MDA seen by other studies when induced by HF diet [54,55].

SOD and catalase constitute two major enzymatic antioxidant defenses against free radical damage in biological system. Reactive oxygen species (ROS) are removed through dismutation of free radicals into H_2O_2 catalyzed by SOD, which is further converted into H_2O by catalase [56]. In this study, we found that HF content seems to decrease plasma SOD activities comparing HF+GTE and LF+GTE, however, the overall postprandial SOD activity was increased compared to baseline in all treatment groups. Indeed, a HF diet was found to compromise the antioxidant defense system. The postprandial blood SOD activity showed a decreasing trend after a HF meal within 2-4 hrs compared to baseline level in exercise-trained healthy subjects [57,58]. However, it should be noted that the subjects in this study consumed a HF meal with potent bioactive compounds, which might in part explain why SOD activity was elevated in all groups. A study showed that postprandial SOD activity responds differently to different diets in subjects. After a meat meal, the plasma SOD remained at a similar level at 2-4 hrs following ingestion, however, after a vegan meal, the plasma SOD activity increased after 2 hrs of intake [59]. In addition, it was found that the postprandial plasma SOD activity increased by 3 fold 4 hrs after a plant-based Mediterranean meal in human subjects [60]. These indicate that the increased SOD activity seen in this study might be due to the antioxidant effect of GTE regardless of fat content.

Following a HF meal, the blood catalase level decreased as a reaction to elevated oxidative stress 2-4 hrs postprandial in healthy exercise-trained subjects [57,58]. Antioxidant enzymes including SOD and catalase were activated after a plant-based Mediterranean meal in human subjects [60]. Long-term consumption of green tea was able to upregulate SOD and catalase enzyme activities, as was previously found in fruit flies [61]. In this study, we found that the postprandial catalase activity following the meal was elevated compared to baseline level regardless of fat content in both LF+GTE and HF+GTE groups. Especially when consumed with LJ, plasma catalase activities tended to increase further. This finding might be due to the antioxidant effect of GTE and the potential synergistic effect of GTE and LJ. However, the acute intake of GTE and LJ did not exert statistically significant antioxidant effects on pigs which can be ascribed to the short exposure time.

In agreement with other findings, plasma SOD and catalase enzyme activities, as first-line defense antioxidants, were positively correlated in this study [62-64]. Additionally, plasma catalase activity was weakly correlated with plasma TG level, which is in accordance with previous findings [65].

The current study demonstrates that a single dose of GTE did not alter oxidative stress markers or lipid profile in 24 hrs and a single dose of lemon juice did not cause a significant improvement in GTE function in the same experimental period in a healthy pig model. However, LJ significantly increased the circulating level of green tea catechin following one-time consumption. This study used a pig model to provide insight into the potential acute antioxidative efficacy of green tea following single dose treatment, and a possibility of synergism of food components to enhance its bioavailability. The doses of GTE and LJ tested in the current study did not cause liver toxicity measured by ALT and AST activity, total bilirubin,

and GGT levels (data not shown). Although acute exposure to HF diet is hypothesized to induce oxidative stress and stress-induced lipogenesis in liver, recent studies in rodent models of acute exposure to HD diet suggest that stress-induced upregulation in lipogenic markers occurs at a time point beyond 72 hrs [66] It is possible that the protective effects of GTE and GTE+LJ on HF-induced hepatic lipogenesis and or injury may be apparent after 72 hrs. Despite the reports on the hepatotoxic side effects of green tea, a recent systematic review of randomized clinical trials suggests that liver-related adverse events are rare with the intake of GTE [67].

Therefore, a future study using higher doses of GTE or LJ is suggested to investigate the reduction in oxidative stress and hyperlipidemia in HF-fed pigs. In addition, longer duration of treatments with GTE and LJ may also lead to more beneficial results.

Conclusions

Our findings revealed that a single dose of GTE had a limited effect on plasma antioxidant enzymes, lipid profile, and lipid peroxidation in HF-fed pigs in 24 hrs. A single dose of LJ combined with GTE did not further influence the action of GTE during the experimental period. Further study with a longer treatment period and larger sample size are warranted to elucidate the potential improved antioxidant efficacy of GTE when combined with LJ.

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Table 5.1. Plasma cholesterol (mg/dL) concentrations after a single dose of LF, HF, LF+GTE, HF+GTE, LF+GTE+LJ, and HF+GTE+LJ in pigs.

Hours post treatment	LF+GTE (n=5)	HF+GTE (n=4)	LF+GTE+LJ (n=3)	HF+GTE+LJ (n=4)
Pre	1.00	1.00	1.00	1.00
1	1.01±0.05	1.10±0.02*	0.92±0.08	0.98±0.06
2	0.94±0.04	0.98±0.04	0.86±0.05	0.99±0.05
3	0.97±0.02	0.92±0.06	0.86±0.06	0.99±0.07
4	0.93±0.02*	0.95±0.06	0.81±0.11	0.95±0.02
6	0.92±0.04	1.06±0.06	0.89±0.11	0.77±0.08*
12	0.87±0.07	0.78±0.09	0.94±0.03	1.16±0.07
24	1.02±0.05	1.01±0.02	0.93±0.04	1.11±0.05

Abbreviation: LF: low fat, HF: high fat, GTE: green tea extract, LJ: lemon juice. Data were calculated as the percentage to the baseline level. * represents a significant difference ($p<0.05$) compared to 0 hr within the group.

Table 5.2. Plasma triglycerides (mg/dL) concentrations after a single dose of LF, HF, LF+GTE, HF+GTE, LF+GTE+LJ, and HF+GTE+LJ in pigs.

Hours post treatment	LF+GTE (n=5)	HF+GTE (n=4)	LF+GTE+LJ (n=3)	HF+GTE+LJ (n=4)
Pre	1.00	1.00	1.00	1.00
1	0.80±0.09*	0.99±0.30	0.73±0.13	0.96±0.10
2	0.99±0.11	0.73±0.14	0.57±0.07*	1.01±0.15
3	1.19±0.12	0.74±0.13	0.54±0.15*	0.85±0.04*
4	0.74±0.22	0.60±0.12*	0.53±0.17	0.68±0.05*
6	0.84±0.07*	0.56±0.07*	0.47±0.04*	0.54±0.17*
12	1.28±0.24	1.68±0.10*	0.55±0.08*	1.42±0.00
24	1.21±0.24	0.94±0.26	0.59±0.07	1.03±0.25

Abbreviation: LF: low fat, HF: high fat, GTE: green tea extract, LJ: lemon juice. Data were calculated as the percentage to the baseline level. * represents a significant difference ($p<0.05$) compared to 0 hr within the group.

Table 5.3. Plasma MDA concentrations (μM) after a single dose of LF, HF, LF+GTE, HF+GTE, LF+GTE+LJ, and HF+GTE+LJ in pigs.

Hours post treatment	LF+GTE (n=5)	HF+GTE (n=4)	LF+GTE+LJ (n=3)	HF+GTE+LJ (n=4)
Pre	1.00	1.00	1.00	1.00
1	1.12±0.16	0.79±0.02*	1.08±0.13	0.60±0.08*
2	1.10±0.16	0.96±0.14	0.93±0.08	0.55±0.15*
3	0.81±0.21	0.84±0.05*	1.09±0.18	0.80±0.15
4	1.05±0.14	0.91±0.11	0.79±0.00	1.17±0.35
6	1.30±0.22	0.85±0.13	0.87±0.08	0.99±0.23
12	1.08±0.17	0.91±0.06	0.95±0.19	0.89±0.34
24	1.10±0.20	0.88±0.18	1.47±0.33	0.99±0.15

Abbreviation: MDA: malondialdehyde, LF: low fat, HF: high fat, GTE: green tea extract, LJ: lemon juice. Data were calculated as the percentage to the baseline level. * represents a significant difference ($p<0.05$) compared to 0 hr within the group.

Table 5.4. Activity of plasma SOD (U/ml) after a single dose of LF, HF, LF+GTE, HF+GTE, LF+GTE+LJ, and HF+GTE+LJ in pigs.

Hours post treatment	LF+GTE (n=5)	HF+GTE (n=4)	LF+GTE+LJ (n=3)	HF+GTE+LJ (n=4)
Pre	1.00	1.00	1.00	1.00
1	1.68±0.18*	1.06±0.36	1.11±0.29	1.15±0.27
2	1.95±0.63	1.14±0.29	1.11±0.44	1.33±0.33
3	1.86±0.46	1.39±0.44	1.51±0.33	1.45±0.49
4	1.97±0.58	1.41±0.37	1.80±1.07	1.30±0.24
6	1.05±0.35	1.83±0.29	0.56±0.35	0.81±0.18
12	2.25±0.51*	1.32±0.48	1.92±0.43	0.89±0.18
24	2.39±0.52*	1.32±0.34	1.93±0.61	1.28±0.06

Abbreviation: SOD: superoxide dismutase, LF: low fat, HF: high fat, GTE: green tea extract, LJ: lemon juice. Data were calculated as the percentage to the baseline level. * represents a significant difference ($p<0.05$) compared to 0 hr within the group.

Table 5.5. Activity of plasma catalase (nmol/min/ml) after a single dose of LF, HF, LF+GTE, HF+GTE, LF+GTE+LJ, and HF+GTE+LJ in pigs.

Hours post treatment	LF+GTE (n=5)	HF+GTE (n=4)	LF+GTE+LJ (n=3)	HF+GTE+LJ (n=3)
Pre	1.00	1.00	1.00	1.00
1	1.08±0.11	1.05±0.13	1.23±0.21	0.93±0.06
2	0.99±0.17	1.06±0.20	1.43±0.69	0.96±0.19
3	0.75±0.17	0.86±0.08	0.74±0.28	0.84±0.09
4	1.24±0.13	1.54±0.53	1.11±0.16	1.15±0.20
6	1.07±0.21	1.21±0.16	1.56±0.47	1.34±0.24
12	1.36±0.27	0.98±0.03	1.40±0.19	1.30±0.07*
24	1.25±0.29	1.11±0.08	1.40±0.40	1.23±0.14

Abbreviation: LF: low fat, HF: high fat, GTE: green tea extract, LJ: lemon juice. Data were calculated as the percentage to the baseline level. * represents a significant difference ($p<0.05$) compared to 0 hr within the group.

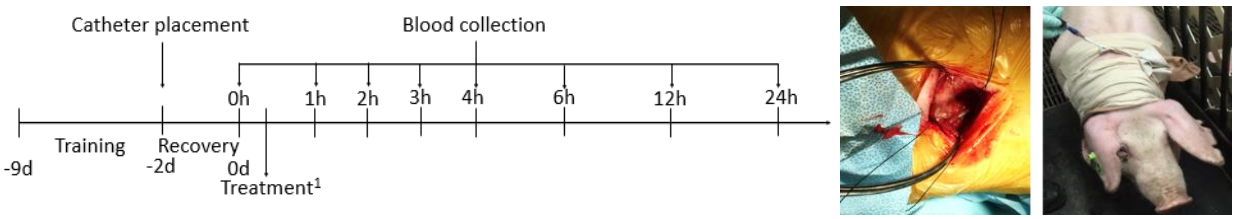

Figure 5.1. Study design. Pigs were provided with either GTE (190mg/kg/treatment) or GTE+L (190mg/kg/treatment GTE +0.75ml/kg lemon juice/treatment) mixed in LF (5% fat diet) or HF diet (22% fat diet). After blood collection at the 0-time point, samples were collected at 1 hr, 2 hrs, 3 hrs, 4 hrs, 6 hrs, 12 hrs, and 24 hrs post-treatment.

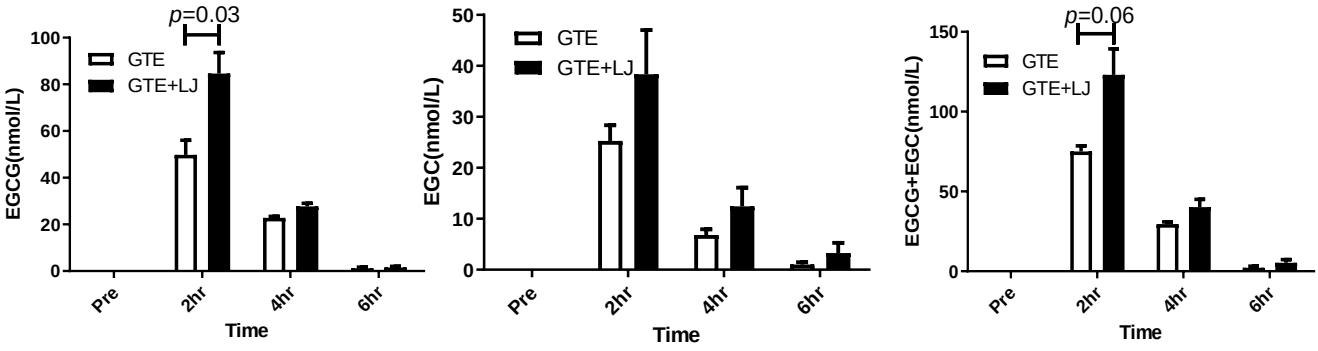

Figure 5.2. Plasma EGCG (a), EGC (b), and EGCG+EGC (c) levels after one-time ingestion of GTE and GTE+LJ with LF diet in pigs (n=3-4).

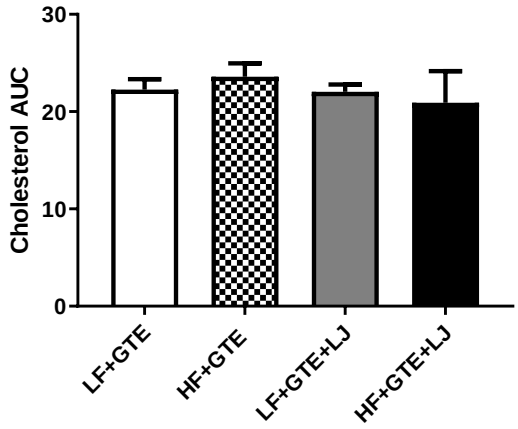

Figure 5.3. Area under curve (AUC) of plasma cholesterol concentration after a single dose of LF, HF, LF+GTE, HF+GTE, LF+GTE+LJ, and HF+GTE+LJ in pigs. Data present means ± SEM; n=3-5 pigs per group. Significance of differences between groups is shown.

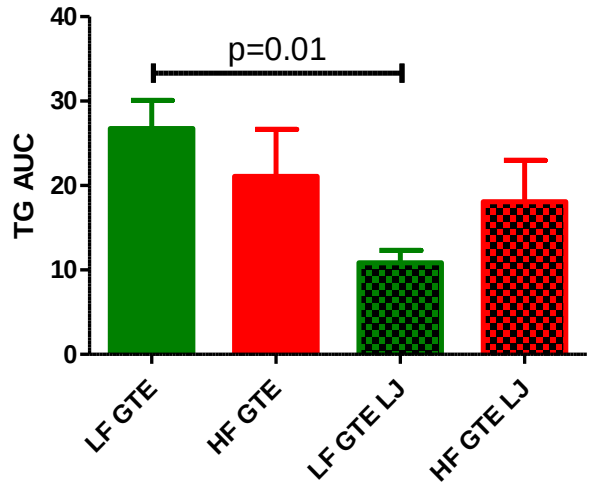

Figure 5.4. Area under curve (AUC) of plasma triglycerides (TG) concentration after a single dose of LF, HF, LF+GTE, HF+GTE, LF+GTE+LJ, and HF+GTE+LJ in pigs. Data present means ± SEM; n=3-5 pigs per group. Significance of differences between groups is shown.

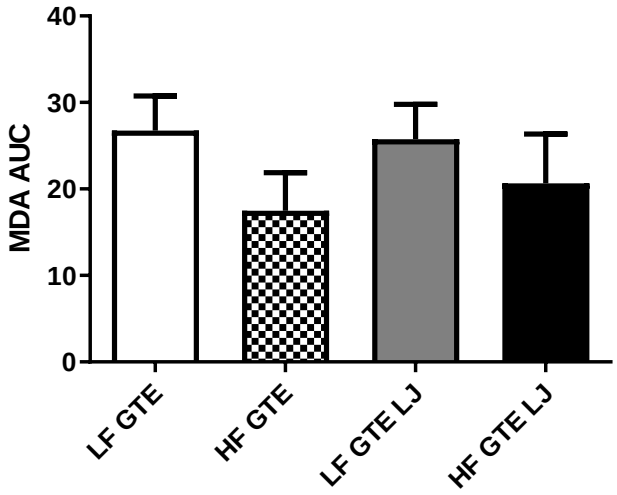

Figure 5.5. Area under curve (AUC) of plasma malondialdehyde (MDA) concentration after a single dose of LF, HF, LF+GTE, HF+GTE, LF+GTE+LJ, and HF+GTE+LJ in pigs. Data present means ± SEM; n=3-5 pigs per group. Significance of differences between groups is shown.

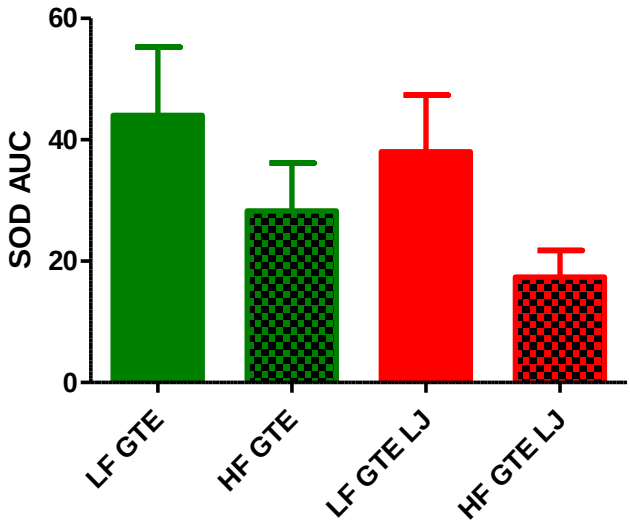

Figure 5.6. Area under curve (AUC) of plasma superoxide dismutase (SOD) activity after a single dose of LF, HF, LF+GTE, HF+GTE, LF+GTE+LJ, and HF+GTE+LJ in pigs. Data present means ± SEM; n=3-5 pigs per group. Significance of differences between groups is shown.

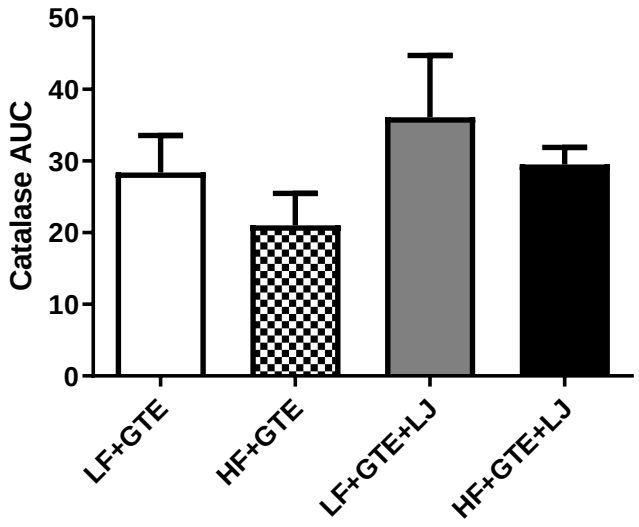

Figure 5.7. Area under curve (AUC) of plasma catalase activity after a single dose of LF, HF, LF+GTE, HF+GTE, LF+GTE+LJ, and HF+GTE+LJ in pigs. Data present means ± SEM; n=3-5 pigs per group. Significance of differences between groups is shown.

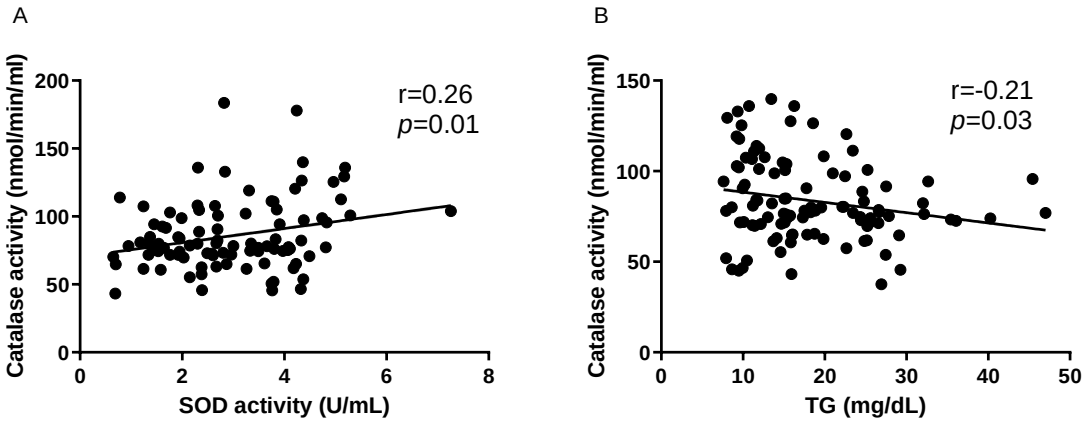

Figure 5.8. Correlation of plasma superoxide dismutase activity and catalase activity (a) and triglycerides (TG) concentration and catalase activity (b) after a single dose of LF, HF, LF+GTE, HF+GTE, LF+GTE+LJ, and HF+GTE+LJ in pigs. Data present means ± SEM; n=3-5 pigs per group. Pearson coefficient (r) and *p*-value is shown.

CHAPTER 6

SUMMARY AND CONCLUSIONS

The objective of this work was threefold: first, to investigate the effects of perinatal DHA supplementation on the neurocognitive function and brain functional organizations in infants, second, to determine the roles of green tea in regulating obesogenic diet-induced metabolic dysfunction, and third, to explore the potentials of improving bioavailability of green tea by co-intake with other bioactive compounds.

The study presented in Chapter 3 was conducted with the primary objective of examining the influence of perinatal DHA supplementation on cognitive performance, myelination, and brain structural and functional organization in piglets. Previously, intervention trials supplementing DHA during gestation and/or lactation in humans yielded mixed and conflicting findings in healthy term infants [1-5]. In this study, DHA supplementation during late gestation and lactation resulted in a remarkably 80-fold increase in the colostrum DHA concentration ($p < .05$) along with the other ω3 PUFA ($p < .05$) without influencing the precursor α-linolenic acid level ($p > .05$). Piglets born to DHA-fed sows demonstrated better cognitive performance in the behavioral testing with more exploratory behaviors in the open field test ($p < .05$) and better object recognition memory in the object recognition test ($p < .05$). In the subsequent inquiry of myelination of the hippocampus, diffusion tensor imaging (DTI) test revealed an increased fiber tract length ($p < .05$) and a trend of increased fractional anisotropy (FA) value ($p = .07$), which is in agreement with the previous findings in which higher DTI indices in the HC was seen in DHA-fed preterm pigs [6]. Finally, we tested brain resting-state networks in piglets at weaning

using functional magnetic resonance imaging (fMRI). Perinatal DHA supplementation resulted in an 8.7% increase of functional connectivity within the cerebellum network, 5.2 % enhanced connectivity within the visual network, 9.8% increase within the default mode network, and a minor increase within the auditory network compared to the control piglets, suggesting that perinatal DHA supplementation may alter brain functional organization of the offspring in healthy piglets. These are the first data that thoroughly examines the effect of perinatal DHA intake on functional and cognitive development of the brain in piglets.

Various bioactive compounds act as important regulators of metabolic processes such as inflammation [7], insulin sensitivity [8], and lipid profile [9]. The studies presented in Chapters 4 was conducted to examine glucose tolerance, insulin sensitivity, and inflammation in the absence of the regulator of G-protein signaling 10 (RGS10), a critical negative regulator of inflammation, and whether green tea extract (GTE) can mitigate these processes in mice fed an obesogenic diet. RGS10 knock-out (KO) mice developed excessive weight gain and adiposity in adipose tissue and liver along with impaired glucose tolerance and insulin sensitivity following a high-fat diet (HFD). Moreover, KO mice augmented inflammation in the liver and adipose tissue following HFD. Importantly, oral consumption of GTE was able to ameliorate weight gain, impaired glucose tolerance, and insulin resistance in KO mice otherwise exaggerated by HFD. This is the first study that demonstrates the protective role of RGS10 in diet-induced insulin resistance and obesity and the beneficial effects of green tea in the absence of RGS10.

Despite the potent health-promoting effects of GTE, the oral bioavailability of green tea catechins is very poor, with most catechins being cleared through the gastrointestinal (GI) tract before making it into circulation [10]. This is suggested to be improved by the co-intake with other bioactive compounds such as citrus juice in vitro [11,12] Therefore, the study presented in Chapter 5

was performed to test the synergistic effect of green tea and lemon juice (LJ) and the antioxidative efficacy of a single dose of GTE in combination of low-fat (LF) or HFD in a pig model. LJ significantly increased the circulating level of green tea catechin following one-time consumption ($p < .05$). However, a single dose of GTE did not alter oxidative stress markers or lipid profile in 24 hrs and the addition of lemon juice did not improve the GTE function in the same experimental period in a healthy pig model. Further study with a longer treatment period and larger sample size are warranted to elucidate the potential improved antioxidant efficacy of GTE when combined with LJ.

In summary, the findings from the studies in Chapters 3, 4, and 5 provide insights into the relationships of bioactive food compounds intake on neurocognitive and metabolic function. While our studies provide evidence for the supportive effects of DHA on the developing brain and green tea on the metabolic functions, further research is warranted to further elucidate the underlying mechanisms of actions with bigger sample size. Findings from these studies deepen the current understanding of the therapeutic roles of bioactive compounds in neural and metabolic regulations that may be applicable to improve brain development and combat obesity-related metabolic syndromes in human population.

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