THE EFFECTS AND MECHANISMS OF CONSUMING SULFUR-CONTAINING DIETARY SUPPLEMENTS ON CHRONIC INFLAMMATION IN ADULTS WITH OBESITY

By

CHANGJIE XU

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2017
To my family
ACKNOWLEDGMENTS

First, I would like to express my sincerely appreciation to my major advisor, Dr. Anne E. Mathews, who generously offered me this fantastic opportunity of entering the PhD program in Nutritional sciences at the University of Florida. Over the past four years, Dr. Mathews has supported me and my research in all the ways possible. I have been so fortunate to have an advisor who gave me the freedom and encouragement to explore my research ideas.

My heartfelt thanks to my co-advisor, Dr. Susan S. Percival, who gave me great opportunity to work on the projects I am presenting here with her and gain research experience in the nutrition field in her laboratory.

I sincerely thank my other committee members, Dr. Bobbi Langkamp-Henken, Dr. Shannon M. Wallet who provided constant help and invaluable suggestions. They helped me achieve the level of knowledge that I possess today.

I would like to express my deepest gratitude to my colleagues, Dr. Cheryl Rowe, Dr. Xiaoshuang Dai, Camila Rodrigues, Brandon Eudy for their assistance and suggestions. I also truly thank all my other colleagues, Dr. Melissa Vilaro, Aseel El Zein, Megan D. Engel, Shannon L. Gordon, Daniel L. Staub, Xiaoyu Zhang for their help and friendship.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>4</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>8</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>9</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>10</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>12</td>
</tr>
<tr>
<td>CHAPTER</td>
<td></td>
</tr>
<tr>
<td>1 LITERATURE REVIEW</td>
<td>14</td>
</tr>
<tr>
<td>Obesity Facts</td>
<td>14</td>
</tr>
<tr>
<td>Epidemiology of Obesity</td>
<td>14</td>
</tr>
<tr>
<td>Costs of Obesity</td>
<td>14</td>
</tr>
<tr>
<td>Obesity-Induced Inflammation</td>
<td>15</td>
</tr>
<tr>
<td>Inflammatory Mediators Involved in Obesity-induced Inflammation</td>
<td>16</td>
</tr>
<tr>
<td>Cellular Mediators Involved in Obesity-induced Inflammation</td>
<td>17</td>
</tr>
<tr>
<td>Mechanisms of Inflammation in Obesity</td>
<td>19</td>
</tr>
<tr>
<td>Oxidative Stress</td>
<td>19</td>
</tr>
<tr>
<td>Adipokines</td>
<td>20</td>
</tr>
<tr>
<td>Endoplasmic Reticulum Stress</td>
<td>21</td>
</tr>
<tr>
<td>Obesity-Induced Inflammation and Obesity Related Diseases</td>
<td>22</td>
</tr>
<tr>
<td>Type 2 Diabetes</td>
<td>22</td>
</tr>
<tr>
<td>Cardiovascular Diseases (CVD)</td>
<td>23</td>
</tr>
<tr>
<td>Cancer</td>
<td>24</td>
</tr>
<tr>
<td>Strategies to Address Obesity-induced Inflammation</td>
<td>25</td>
</tr>
<tr>
<td>Physical Activity</td>
<td>25</td>
</tr>
<tr>
<td>Anti-inflammatory Drugs</td>
<td>26</td>
</tr>
<tr>
<td>Diet</td>
<td>27</td>
</tr>
<tr>
<td>Summary</td>
<td>28</td>
</tr>
<tr>
<td>2 INTRODUCTION AND RESEARCH OBJECTIVES</td>
<td>31</td>
</tr>
<tr>
<td>Background</td>
<td>31</td>
</tr>
<tr>
<td>Objectives and Hypotheses</td>
<td>32</td>
</tr>
<tr>
<td>Summary</td>
<td>33</td>
</tr>
<tr>
<td>3 AGED GARLIC EXTRACT SUPPLEMENTATION MODIFIES INFLAMMATION AND IMMUNITY OF ADULTS WITH OBESITY: A RANDOMIZED, DOUBLE-BLIND, PLACEBO-CONTROLLED CLINICAL TRIAL</td>
<td>34</td>
</tr>
</tbody>
</table>
INTRODUCTION

Study Design and Methods ................................................................. 35
Participations and Study Design .......................................................... 35
Study Procedures ................................................................................. 36
Separation and Culture of PBMC .......................................................... 38
Immune Cell Distribution within the Total PBMC Population .................. 38
Total GSH and C-reactive Protein Concentrations Determination ............... 39
Inflammatory Cytokine Determination ................................................. 39
Statistical Analysis ............................................................................. 39

Results .................................................................................................. 40
Baseline Demographic Characteristics ................................................... 40
Intervention Compliance ...................................................................... 40
Immune Cell Distribution ..................................................................... 41
Markers of Inflammation ..................................................................... 41
Clinical Parameters ............................................................................ 42

Discussion ............................................................................................ 42
AGE and Inflammatory Mediators During Obesity-induced Inflammation ..... 43
AGE and Immune Cell Populations During Obesity-induced Inflammation ... 44
AGE and Risk Factors of Cardiovascular Diseases .................................. 45
Strength and Limitations .................................................................... 46
Summary ............................................................................................... 47

4 ORAL SUPPLEMENTATION WITH 500 mg GSH DOES NOT MODIFY OBESITY-
INDUCED INFLAMMATION IN OLDER ADULTS: A RANDOMIZED, DOUBLE-
BLIND, PLACEBO-CONTROLLED CLINICAL TRIAL ....................................... 56

Introduction .......................................................................................... 56
Study Design and Methods ...................................................................... 57
Study Design and Participants ............................................................... 57
Sample Size Determination .................................................................... 57
Study Procedure ..................................................................................... 58
Anthropometric and Biochemical Measurements ....................................... 59
Separation and Culture of PBMC .......................................................... 59
Immune Cell Distribution within the Total PBMC Population .................. 59
Total GSH ............................................................................................. 60
Inflammatory Cytokine Determination ................................................. 60
Statistical Analysis .............................................................................. 60

Results .................................................................................................. 61
Baseline Participant Demographic Characteristics ................................... 61
Compliance ......................................................................................... 61
Immune Cell Distribution .................................................................... 61
Respiratory Burst ................................................................................ 61
Markers of Inflammation ..................................................................... 62
Clinical Parameters ............................................................................. 62

Discussion ............................................................................................ 62
GSH Supplementation and Intracellular GSH .......................................... 62
GSH Supplementation and Immune Cell Populations .............................. 63
5 AGED GARLIC EXTRACT AND GSH REDUCE LPS-INDUCED INFLAMMATION IN ADIPOCYTES THROUGH INHIBITING ACTIVATION OF NF-κB AND MAPK PATHWAYS ......................................................75

Introduction ..................................................................................................................75
Materials and Methods .................................................................................................76
   Aged Garlic Extract and GSH Solution Preparation ....................................................76
   Cell Culture and Differentiation ..................................................................................77
   Study Design ..............................................................................................................77
   Inflammatory Mediators Determination ......................................................................78
   Total GSH Concentration Determination ....................................................................78
   RNA Extraction and Quantitative Real-time PCR ......................................................78
   Immunoblotting .........................................................................................................78
   Statistical Analysis ....................................................................................................80
Results ..........................................................................................................................80
   Inflammatory Mediators Secreted by Differentiated 3T3-L1 Cells ..............................80
   mRNA Expression Levels of Cytokines in Differentiated 3T3-L1 Cells .......................80
   Intracellular GSH Concentration ..............................................................................81
   Expression Levels of AP1 and NF-κB .......................................................................81
Discussion ....................................................................................................................81
   AGE and GSH Prevented LPS-induced Inflammation in 3T3-L1 Cells .......................81
   AGE and GSH Inhibited Activation of NF-κB and AP1 .............................................82
   Strengths and Limitations .........................................................................................83
Summary ......................................................................................................................83

6 CONCLUSIONS AND FUTURE WORK SUMMARY ....................................................92

Conclusions .................................................................................................................92
Strengths and Limitations ..........................................................................................94
Future Direction .........................................................................................................95

LIST OF REFERENCES ...............................................................................................96

BIOGRAPHICAL SKETCH .........................................................................................111
<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>Inflammatory cytokines in obesity ................................................................. 29</td>
</tr>
<tr>
<td>3-1</td>
<td>Demographics and baseline clinical characteristics of study participants at baseline. ...... 48</td>
</tr>
<tr>
<td>3-2</td>
<td>Other characteristics of participants .................................................................. 49</td>
</tr>
<tr>
<td>3-3</td>
<td>Immune cell distribution at baseline and completion of the study ......................... 50</td>
</tr>
<tr>
<td>3-4</td>
<td>Serum biomarkers at baseline and completion of the study .................................... 51</td>
</tr>
<tr>
<td>3-5</td>
<td>Cytokine secreted by PBMC after 24 h stimulation of PHA-L .................................... 52</td>
</tr>
<tr>
<td>4-1</td>
<td>Demographics and baseline clinical characteristics of study participants .................. 66</td>
</tr>
<tr>
<td>4-2</td>
<td>Self-reported side effects, illnesses, and related behaviors of participants throughout the intervention period. ............................................................................................................ 67</td>
</tr>
<tr>
<td>5-1</td>
<td>Sequences of primers used for real-time quantitative PCR ........................................ 84</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
</tr>
<tr>
<td>1-1</td>
<td>Prevalence of obese in the United States.</td>
</tr>
<tr>
<td>3-1</td>
<td>Overall flow of participants assessed for eligibility, enrolled in the AGE study, randomized to different treatments and analyzed statistically.</td>
</tr>
<tr>
<td>3-2</td>
<td>Participant compliance.</td>
</tr>
<tr>
<td>4-1</td>
<td>Overall flow of participants assessed for eligibility, enrolled in the GSH study, randomized to different treatments and analyzed statistically.</td>
</tr>
<tr>
<td>4-2</td>
<td>Participant compliance.</td>
</tr>
<tr>
<td>4-3</td>
<td>The percentage of immune cell populations in the total peripheral blood mononuclear cell among the 4 groups at baseline and the end of the study.</td>
</tr>
<tr>
<td>4-4</td>
<td>Respiratory burst assay was performed to quantify a respiratory burst response in neutrophils and monocytes by flow cytometry.</td>
</tr>
<tr>
<td>4-5</td>
<td>Cytokines secreted by PBMC after 24 h stimulation with PHA-L among the 4 groups at baseline and the end of the study.</td>
</tr>
<tr>
<td>4-6</td>
<td>Blood clinical parameters among the 4 groups.</td>
</tr>
<tr>
<td>4-7</td>
<td>Anthropometric measurements among the 4 groups at baseline and the end of the study.</td>
</tr>
<tr>
<td>5-1</td>
<td>Free fatty acids induce inflammation in obesity.</td>
</tr>
<tr>
<td>5-2</td>
<td>Concentrations of cytokines secreted by differentiated 3T3-L1 cells in cell culture medium.</td>
</tr>
<tr>
<td>5-3</td>
<td>mRNA expression levels of Adiponectin, IL-6, TNF-α and MCP-1 genes in differentiated 3T3-L1 cells.</td>
</tr>
<tr>
<td>5-4</td>
<td>Concentrations of intracellular GSH in differentiated 3T3-L1 cells.</td>
</tr>
<tr>
<td>5-5</td>
<td>Expression of NF-κB in nucleus and cytosol of 3T3L1 cells.</td>
</tr>
<tr>
<td>5-6</td>
<td>Expression of activator protein 1 in nucleus and cytosol of 3T3L1 cells.</td>
</tr>
<tr>
<td>5-7</td>
<td>Potential mechanism by which AGE and GSH reduces obesity-induced inflammation.</td>
</tr>
</tbody>
</table>
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGE</td>
<td>Aged garlic extract</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>API</td>
<td>Activator protein 1</td>
</tr>
<tr>
<td>ATF6</td>
<td>Activating transcription factor 6</td>
</tr>
<tr>
<td>ATMs</td>
<td>Accumulation of adipose tissue macrophages</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>CCR2</td>
<td>C-C motif chemokine receptor 2</td>
</tr>
<tr>
<td>CHD</td>
<td>Coronary artery diseases</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular diseases</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic acid</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>eIF4E</td>
<td>Eukaryotic translation initiation factor 4E</td>
</tr>
<tr>
<td>EPA</td>
<td>Eicosapentaenoic acid</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinases</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FFAs</td>
<td>Free fatty acids</td>
</tr>
<tr>
<td>GLU</td>
<td>Glucose</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein cholesterol</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>IRS-1</td>
<td>Insulin receptor substrate-1</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinases</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein cholesterol</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein-1</td>
</tr>
<tr>
<td>MIF</td>
<td>Macrophage migration inhibitory factor</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NK cell</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>NKT cell</td>
<td>Natural killer T cell</td>
</tr>
<tr>
<td>non-HDL</td>
<td>Non-high density lipoprotein cholesterol</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PERK</td>
<td>Protein kinase RNA-like endoplasmic reticulum kinase</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acids</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SAD</td>
<td>Sagittal abdominal diameter</td>
</tr>
<tr>
<td>TC</td>
<td>Total cholesterol</td>
</tr>
<tr>
<td>TCA cycle</td>
<td>Tricarboxylic acid cycle</td>
</tr>
<tr>
<td>TG</td>
<td>Triglyceride</td>
</tr>
<tr>
<td>Th cell</td>
<td>T helper cell</td>
</tr>
<tr>
<td>TLR4</td>
<td>Toll like receptor 4</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-alpha</td>
</tr>
<tr>
<td>Treg cell</td>
<td>Regulatory T cell</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
</tr>
<tr>
<td>WC</td>
<td>Waist circumference</td>
</tr>
</tbody>
</table>
THE EFFECTS AND MECHANISMS OF CONSUMING SULFUR-CONTAINING DIETARY SUPPLEMENTS ON CHRONIC INFLAMMATION IN ADULTS WITH OBESITY

By

Changjie Xu

December 2017

Chair: Anne Mathews
Major: Nutritional Sciences

Obesity is a global health issue. In the United States, more than one-third of adults are obese (1). Obesity induces chronic systemic inflammation and increasing obesity positively correlates with the concentration of inflammatory cytokines (2, 3). Obesity-induced inflammation is recently considered a link between obesity and its related chronic diseases (4). Prevention or reduction of inflammation in adults with obesity may mitigate the associated negative health complications such as cardiovascular disease and some cancers.

Increased oxidative stress in obesity plays a key role in the development of inflammation. Overproduced reactive oxygen species (ROS) stimulate the activation of nuclear factors such as NF-κB and activator protein (AP1), which can up-regulate the production of inflammatory cytokines (5). Glutathione (GSH), one of the most abundant ROS scavengers in the body, protects host immune cells from free radical assault. GSH is diminished with obesity (6, 7). Daily consumption of dietary supplements that support GSH production may be a safe and acceptable mode of counteracting the chronic inflammation associated with obesity.

The goal of this research was to evaluate the effects and mechanisms of sulfur-containing dietary supplements on chronic inflammation in adults with obesity. To achieve this goal, three
independent studies were conducted, including two randomized placebo-controlled clinical trial and one cell culture based experiment. The dietary supplements tested in the clinical trials were Aged garlic extract (AGE) (Wakunaga of America Co.) and Setria® GSH (Kyowa Hakko Bio Co.). Six weeks of AGE consumption (3.6 g/day) modulated immune cell distribution, attenuated increase of serum TNF-α (p = 0.04) and IL-6 (p = 0.05) concentrations and reduced blood LDL (p = 0.05) concentration in adults with obesity, indicating that the consumption of AGE can produce anti-inflammatory effects in adults with obesity. Similar the anti-inflammatory effects were not observed when adults consumed GSH (500 mg/day) for four months. Both AGE and GSH were used to pre-treat differentiated adipocyte cells (3T3-L1) prior to stimulation of inflammation with lipopolysaccharides (LPS). Pre-treatment with both AGE and GSH, decreased the expression of IL-6 in protein and at the mRNA level. AGE pre-treatment also increased the expression of adiponectin in the cells. Moreover, AGE and GSH pre-treatment inhibited activation of NF-κB and AP 1, two major nuclear factors that are associated with inflammation.

Taken together, this series of experiments support the hypothesis that sulfur-containing supplements may reduce the inflammation in obesity. The in vitro study suggested that the anti-inflammatory effect is through the inhibition of the activation of NF-κB and MAKP pathways. This anti-inflammatory effect was only observed in the AGE clinical study, but not when participants were provided GSH. Further studies should be conducted to determine the optimal dose of unique sulfur-containing supplements for reducing obesity-induced inflammation.
CHAPTER 1
LITERATURE REVIEW

Obesity Facts

Obesity is a serious worldwide health issue, especially in western countries. Recently, it has received considerable attention from health professionals and researchers. According to the World Health Organization (WHO), obesity, or excess adiposity, is defined as having a body mass index (BMI) greater than 30.0 kg/m².

Epidemiology of Obesity

The overall prevalence of obesity has increased rapidly in the last few decades. It more than doubled between 1980 and 2014. In 2014, about 13% of the world’s adult population (11% of men and 15% of women) were obese, compared with 4.8% of men and 7.9% of women in 1980 (8). In the United States, according to reports from the Centers for Disease Control and Prevention, about 36.5% of American adults were obese in 2016 (1). There is a significant increasing linear trend in prevalence of obesity among adults and youth from 1999-2000 through 2015-2016 (Figure 1-1 B). In England, the prevalence of obesity among adults has increased from 15% in 1993 to 26% in 2014. The rate of increase has slowed down since 2001, although the trend is still upwards (9). In China, the prevalence of age-adjusted obesity in adults (≥ 20 years) was 11.3% in 2011 (11.8% of men and 11.0% of women), compared with 2.88% of men and 4.5% of women in 1991 (10). Although the prevalence is relatively low in China, it has increased dramatically in the last few years.

Costs of Obesity

Excess adiposity harms health in many ways. It increases the risk of developing cardiovascular diseases, diabetes, cancers and some other chronic diseases. Billions of dollars are spent on treating obesity and obesity-related conditions every year. There are two types of costs
that are associated with the treatment of obesity and obesity-related conditions: direct costs and indirect costs. Direct costs are those from preventive, diagnostic, and therapeutic services. Indirect costs are economic losses due to obesity related morbidity and mortality. In United States, annual medical costs related to obesity were $147 billion in 2008. And medical costs for people who are obese were $1,429 higher than those of normal weight (11). In England, estimates of the direct costs for treating obesity and related conditions was £4.2 billion in 2007 (12), compared with £479.3 million in 1998 (13). The indirect costs increased from £2.6 billion in 1998 (13) to £15.8 billion in 2007 (12). In Scotland, the total medical cost was estimated to be £600 million for obesity and £1.4 billion for overweight in 2007-2008 (14).

The high prevalence of obesity in the worldwide and the costs associated with obesity demonstrate the enormous global burden of obesity. Researchers are actively seeking ways to better understand the physiological effects of obesity and how to reduce the negative impact excess adiposity has on health. A most recent area of focus is on understanding obesity-induced inflammation.

**Obesity-Induced Inflammation**

In 1993, Hotamisligil et al. demonstrated that adipose tissue of obese mice expressed tumor necrosis factor-alpha (TNF-α), which was associated with insulin resistance in those mice (15). Since then, plenty of researchers have focused on this area and have demonstrated that obesity is often associated with a state of chronic low-grade inflammation, called obesity-induced inflammation. The development and progression of this chronic inflammation is a rather complicated process. It involves many molecular and cellular responses.
Inflammatory Mediators Involved in Obesity-induced Inflammation

It is well known that adipose tissue is one of the dominant sites in the body for production and release of cytokines. One of the major characteristics of obesity is the expansion of adipocytes. Thus, these expanded adipocytes elevate production of pro-inflammatory cytokines and cause chronic systemic inflammation. Significant changes in certain inflammatory markers have been observed in obesity, including C-reactive protein (CRP), inflammatory cytokines and adipokines (Table 1-1).

CRP is an acute-phase protein, synthesized by the liver. It increases rapidly in response to inflammation. Thus, it is usually used as a marker of inflammation. The serum concentration of CRP has been found positively correlated with obesity, even in young adults (16). A systematic review (17) analyzed the association between obesity and CRP in a meta-analysis of 51 cross-sectional studies. Serum CRP was found to positively associate with obesity in various populations. However, this association was found to be stronger in women than men, and in people from western countries compared with people from Asian countries. The reasons for these differences are not well understood.

Following the elevation of CRP concentration in the body, macrophages and T cells will increase the secretion of inflammatory cytokines during obesity-induced inflammation. Studies have shown that body fatness, represented by both BMI and waist circumference (WC) is significantly correlated with serum concentrations of inflammatory cytokines (3, 18). There are two kinds of inflammatory cytokines, pro-inflammatory cytokines and anti-inflammatory cytokines. In obese individuals, concentrations of pro-inflammatory cytokines, interleukin 6 (IL-6) (19) and TNF-α (20), are significantly higher than those found in lean individuals. And serum
concentrations of pro-inflammatory cytokines are positively associated with weight, BMI, WC and hip circumference (3).

Another type of inflammatory marker altered in obesity is adipokines. Adipokines are secreted by adipocytes. The two major adipokines discussed here are leptin and adiponectin. Compared with adipose tissue of lean adults, the concentration of leptin is greater in adipose tissue of obese adults, while the concentration of adiponectin is decreased. Leptin is an adipose-derived hormone that plays an important role in regulating energy intake and expenditure. It also regulates immune function by stimulating production of macrophages, involving in activation of natural killer cell and inducing secretion of pro-inflammatory cytokines (21, 22). In obesity, leptin is increased by as much as four-fold compared with lean adults and this difference is especially noted in women (23, 24). Adiponectin is another adipose-derived hormone, which is involved in regulating glucose and fatty acid metabolism. It is an anti-inflammatory adipokine. There are at least two kinds of receptors for adiponectin, ADIPOR1 and ADIPOR2, on the cell surface. Compared with lean adults, adiponectin concentrations are decreased in adults with obesity (24). Yang et al. investigated plasma adiponectin concentration in overweight and obese Asians and found that an inverse correlation with BMI (25).

**Cellular Mediators Involved in Obesity-induced Inflammation**

Besides the inflammatory mediators, a large number of cells are also involved in obesity-induced inflammation.

Adipocytes, the predominant component of adipose tissue, are usually known to play roles in the metabolic pathway. Until recently, its role in inflammatory pathway was broadly studied, and still not well understood. In an obese state, not only do the adipocytes increase in number and size to accumulate fat, they also secrete IL-6, monocyte chemoattractant protein
MCP-1) and adipokines. Curat et al. demonstrated that increased secretion of adipokines from mature human adipocytes promoted the infiltration and accumulation of macrophages in adipose tissue (26), leading to development of the low grade inflammation. Release of those inflammatory mediators secreted by adipocytes into circulation also contribute to obesity-induced inflammation.

Accumulation of adipose tissue macrophages (ATMs) plays a critical role in the development of obesity-induced inflammation. There are two types of macrophage phenotypes, pro-inflammatory M1 type and anti-inflammatory M2 type. During obesity, the predominant phenotype of ATMs switches from M2 to M1 (27). Fujisaka et al. observed that both the number of M1 macrophage and the ration of M1 to M2 increased in epididymal fat tissue of high fat diet-induced obese mice (28). Macrophages from obese mice overexpress genes that are related with inflammation, including those encoding interleukin-6, TNF-α and C-C motif chemokine receptor 2 (CCR2), whereas macrophages from lean mice express more anti-inflammatory genes such as interleukin 10 (27, 29). As obesity progresses, more M1 ATMs were recruited into adipose tissue, and secreted inflammatory cytokines which further promote obesity-induced inflammation.

Not only adipose tissue macrophages, but other innate immune cells are also impaired with the progression of obesity. Natural killer (NK) cells represent the first line of the immune system. Studies have shown decreased circulating NK cells in obesity (30, 31). However, when separated from metabolically unhealthy obese patients, NK cell counts in metabolically healthy obese subjects was not different from the lean subjects (31, 32). Neutrophils, a member of the granulocytes family, are clinically used as evidence of microbial infection. Talukdar et al. found that the number of neutrophils numbers increased in adipose tissue of HFD-induced obesity mice.
Those elevated neutrophils produce chemokines and cytokines, promoting macrophage infiltration into adipose tissue, which may contribute to development of obesity-induced inflammation.

T cells are related to obesity as well. T cells that express the surface marker CD4 are T helper (Th) cells which are usually divided into two populations: the pro-inflammatory Th1 cells and anti-inflammatory Th2 cells. Another CD4+ population, regulatory T cells (Treg), can secrete anti-inflammatory cytokine to suppress activation of the immune system and prevent autoimmune attack. In adipose tissue, Treg cells inhibit macrophage migration and promote M2 macrophage phenotype. T cells that express the surface marker CD8 are cytotoxic T cells, which secrete pro-inflammatory cytokines. As obesity progresses, cytotoxic T cells are activated in adipose tissue, leading to the recruitment and activation of ATMs (34). Infiltration of cytotoxic T cells into adipose tissue is also associated with the deletion of adipose Treg cells and increased activation of Th1 cells (34, 35). γδ-T cells, a subset of T cells expressing γδ-TCRs, were recently found to be increased in adipose tissue and may associated with obesity-induced inflammation during obesity (36).

**Mechanisms of Inflammation in Obesity**

As mentioned above, the development of obesity-induced inflammation is a complicated process. It involves many molecular and cellular responses. The exact mechanism of how the low-grade inflammation is induced is not clear yet. Several potential mechanisms have recently been proposed recently.

**Oxidative Stress**

Increased oxidative stress was observed in adults with obesity (37), and there is a positive association between oxidative stress (8-epi-PGF$_{2\alpha}$ as a marker) and BMI (38). Under an obese
condition, the excessive fat accumulation leads to increased generation of electron donors, NADH and FADH2, by β-oxidation of fatty acids and the oxidation of FFA-derived acetyl CoA by the tricarboxylic acid (TCA) cycle. NADH and FADH2 are processed in the mitochondrial electron transport chain for electron donation to oxygen, finally leading to increased production of reactive oxygen species (ROS) and oxidative stress (39). ROS can then activate the transcription factor nuclear factor kappa B (NF-κB) signaling pathway (40), and mitogen-activated protein kinase (MAPK) pathway (41), both of which play important roles in obesity-induced inflammation. In obese individuals, the elevated ROS activate IKK complex which, in turn, phosphorylates IκBα. After phosphorylation, IκBα disassociates with NF-κB and is polyubiquitinated, leading to its degradation by the proteasome and to translocation of the NF-κB dimer to the nucleus (42, 43), and finally an increase in the expression of pro-inflammatory genes.

**Adipokines**

The increase of leptin and decrease of adiponectin plays an important role in the development of obesity-induced inflammation. The overexpressed leptin binds to its receptors and activates the canonical JAK-STAT, extracellular signal-regulated kinases (ERK) and Phosphoinositide 3-kinase signaling pathway by JAK-mediated tyrosine phosphorylation of the intracellular domain (44), which stimulates secretion of the pro-inflammatory cytokines, including leukotriene B4, TNF, IL-1 and IL-6 (45, 46). It also indirectly activates eukaryotic translation initiation factor 4E (eIF4E) by inhibiting eIF4E-Binding Protein 1. Activated eIF4E will induce perilipin-2 expression, lipid accumulation and foam cell formation in macrophages (44). Finally, stimulates the production of pro-inflammatory cytokines.
In contrast to leptin, when adiponectin binds to its receptors, it suppresses production of pro-inflammatory factors, like TNF and interferon and induces secretion of anti-inflammatory cytokines, like IL-10 and IL-1RA (47) by activation of AMP-activated protein kinase and p38 MAPK. It also activates peroxisome proliferator-activated receptors (PPARs). PPARs in turn inhibit the transcriptional activation of pro-inflammatory response genes (48), including TNF-α, IL-6, cyclooxygenase-2, vascular cell adhesion molecule-1, inducible NO synthase and matrix metalloproteinase (49-51).

**Endoplasmic Reticulum Stress**

Another key contributor to obesity-induced inflammation is endoplasmic reticulum (ER) stress, which is usually caused by the unfolded protein response (UPR) (52). UPR is induced primarily by activation of three transmembrane proteins residing on the ER, protein kinase RNA-like endoplasmic reticulum kinase (PERK), inositol requiring enzyme 1, and activating transcription factor 6 (ATF6). Those three pathways are tightly connected with inflammatory signaling pathways. PERK activation can lead to enhanced NF-κB signaling. PERK, as a kinase, can inhibit general protein translation by the phosphorylating alpha subunit of eukaryotic initiation factor 2α, which in turn decreases translation of IκBα. IκBα is a negative regulator of IKK and NF-κB signaling. The inhibition of IκBα leads to enhanced activation of NF-κB. The consequence is increased expression of the pro-inflammatory genes in the downstream of NF-κB, like TNF-α and IL-6 (53). IRE-1 can upregulate the expression of pro-inflammatory cytokines through their interaction with TNF receptor-associated factor 2, which is required for TNF-α mediated activation of c-Jun N-terminal kinases (JNK) and NF-κB (54-56). ATF-6 has also been shown to increase NF-κB signaling through activation of Akt (57).
Neeraj et al. reported that the expression of ER stress markers genes, including \textit{HSPA5}, \textit{ATF6a}, and \textit{EIF2AK3}, and downstream chaperones was positively correlated with BMI and percent of body fat (58). The increased ER stress in obese individuals plays a crucial role in the development of obesity-induced inflammation through activation of the NF-κB signaling pathway.

**Obesity-Induced Inflammation and Obesity Related Diseases**

It has been well accepted that overweight and obesity are related to many chronic diseases. Overweight and obesity have adverse metabolic effects on blood pressure, cholesterol, triglycerides and insulin sensitivity. BMI is positively related to risks of coronary heart disease, ischemic stroke and type 2 diabetes mellitus. In addition, overweight and obesity also increases the risks of cancer of the breast, colon, gastric cardia, pancreas, kidney and gall bladder (59, 60). This chronic low-grade inflammation is considered the key link between obesity and its related chronic diseases (4).

**Type 2 Diabetes**

One of the most common diseases associated with obesity is type 2 diabetes. Type 2 diabetes is characterized by insulin resistance, a condition in which insulin receptors on the surface of cells can not recognize insulin, leading to hyperglycemia. It is well established that obesity-induced inflammation plays a crucial role in the development of insulin resistance (61). As obesity progresses, some pro-inflammatory cytokines secreted by adipose tissue likely contribute to the pathogenesis of insulin resistance (62). Those cytokines can inhibit insulin action by activating serine kinases, including \textit{IκB} kinase and JNK, which phosphorylate serine residues of the insulin signaling pathway (63). In normal conditions, when insulin binds to its receptors, it will activate an insulin receptor substrate-1 (IRS-1), an essential medium in the
insulin signaling cascade by tyrosine phosphorylation. This phosphorylation enables IRS-1 to recruit downstream proteins and develop a scaffolding network. One of these downstream proteins is HMS untamed protein (58) which can activate PI3-kinases and finally reduce blood glucose concentration by stimulating translocation of glucose transport 4 (GLUT4) to the cell surface (64). In the insulin resistant state, serine phosphorylation of IRS-1 will inhibit its action and stop its initial signaling (65). TNF-α is the first inflammatory mediator linking insulin resistance with obesity (15), by inhibiting tyrosine kinase activity of insulin receptors. The serum concentration of TNF-α in obese adults is much higher than those of normal weight adults (66). A mice study showed that deletion of TNF-α or TNF receptor 1 protected the body from insulin resistance (67). Other cytokines secreted by obese adipose tissue that can interfere insulin signaling, are IL-1α (68), IL-1β (69), IL-6 (69, 70), and macrophage migration inhibitory factor (MIF) (71), etc.

**Cardiovascular Diseases (CVD)**

Obesity has been identified as an independent risk factor for CVD, including coronary heart diseases (CHD), stroke, heart failure, hypertensive heat diseases, etc. (72). Rimm et al. reported that men with a BMI of 25 to 28.9 kg/m², are 1.72 times more likely to have CHD than those with BMI less than 23.0 kg/m² (73). The risk increased to 2.61 times with a BMI of 29.0 to 32.9 kg/m² and 3.44 times with a BMI of 33.0 or greater. The association of obesity and coronary heart disease is also observed among women (74). As among men, the risk of CHD is positively correlated with BMI, with 3.3 times higher risk with a BMI of 29.0 kg/m² or greater (74). Kenchaiah et al. showed that obesity increased the risk of heart failure as well. In their study, the risk of heart failure doubled among obese subjects, with a hazard ratio of 2.12 for women and 1.90 for men (75).
Atherosclerosis, the primary underlying cause of cardiovascular disease, was thought to be a mild lipid storage disease (76). Now, there is a better understanding of the mechanisms for atherosclerosis. Studies have shown that inflammation is closely related to the development of atherosclerosis, from the initiation to the end-stage thrombotic complications (77). In obese individuals, macrophages accumulate within the adipose tissue (78), leading to production of several pro-inflammatory mediators, including IL-1, IL-6 and TNF-α. As excess nutritional stress continues, it creates a pro-inflammatory environment in the body. Stimulated by inflammatory cytokines, endothelial cells express adhesion molecules which allow leukocytes to adhere to the artery wall (79), and finally result in endothelial dysfunction which is responsible for the pathophysiological changes in subclinical atherosclerosis and CVD (80).

Cancer

Large numbers of studies have reported a positive relationship between obesity and cancers. Ma et al. systematically reviewed 54 prospective studies on obesity and risk of colorectal cancer and showed that the risk of colorectal cancer in obese subjects was 1.3 times more than those with normal weight (81). Chen et al. reported a positive association between excess body weight and primary liver cancer (82). In a systematic review of 14 cohort studies, BMI was also found positively associated with pancreatic cancer with a risk ratio of 1.30 comparing subjects with BMI of 25 kg/m² or greater to those with BMI less than 25 kg/m².

Chronic inflammation is critical for progression of tumor growth. Inflammatory cytokines secreted during the chronic low-grade inflammation in subjects with obesity can influence growth, mutation, proliferation, differentiation and movement of cells through DNA damage, angiogenesis and actions as growth factors (83). TNF-α, one of the major mediators secreted during obesity, was found to act as a tumor promoter via activation of NF-κB (84) and
to damage DNA by increasing reactive oxygen species levels (85). Jaiswal et al. treated three human cholangiocarcinoma cell line with a mixture of IL-1β, IFN-γ and TNF-α (86). Results showed that inflammatory cytokine induced production of inducible NO synthase (iNOS) and NO. NO in turn damaged DNA directly and indirectly by inactivating DNA repair proteins. Inflammatory cytokines also inhibit tumor-suppressor protein p53 activity. Cells treated with MIF showed inhibition of p53 activity (87), leading to proliferation, differentiation and migration of tumor cells. In addition, some inflammatory cytokines act as growth factors for tumor cells. IL-6 is a growth factor for multiple myeloma cells (88). It stimulates release of parathyroid-hormone related protein from these cells, induces secretion of TNF-related activation-induced cytokine (TRANCE) and finally leads to cell growth.

**Strategies to Address Obesity-induced Inflammation**

As obesity-induced inflammation increases one’s risk of many chronic diseases. It is important to find appropriate strategies to address this problem.

**Physical Activity**

Regular and chronic physical activity is an effective method for addressing obesity-induced inflammation. It can decrease production of inflammatory markers and increase anti-inflammatory substances. Studies have shown that there is an inverse correlation between regular exercise and the serum concentrations of inflammatory biomarkers, such as CRP, IL-6, and TNF-α (89, 90). Starkie et al. reported that physical exercise was even able to inhibit endotoxin-induced TNF-α production in humans (91).

The anti-inflammatory function of physical activity is not only represented by decreased secretion of pro-inflammatory cytokines, but also increased expression of antioxidant and anti-inflammatory mediators (92, 93). Kriketos et al. reported that 2-3 sessions of regular moderate
exercise could remarkably increase adiponectin concentration in serum (94). This effect could be sustained up to 10 weeks. Those findings were supported by Simpson et al. who systematically reviewed 8 randomized controlled trials over ten years (95). In another study, subjects with type 2 diabetes enrolled in a 12-week aerobic training program demonstrated an up-regulated expression of superoxide dismutase (SOD) and catalase (CAT), which are two important antioxidant enzymes in the body (96).

**Anti-inflammatory Drugs**

Anti-inflammatory drugs are another choice. Aspirin, one of the most commonly used anti-inflammatory medications was found to improve blood glucose level in individuals with type 2 diabetes as early as 1901 (97). Kim (98) and Yuan (99) et al. demonstrated that aspirin also could improve insulin resistance and decrease inflammation. Hundal et al. supported those findings in a human study (100). Here, they provided participants with diabetes with high dose aspirin (approximately 7g/day) for 2 weeks and observed a 25% reduction in fasting plasma glucose, 15% reduction in total cholesterol and C-reactive protein, a 50% reduction in triglycerides, and a 30% reduction in insulin clearance.

As described above, TNF-α was the first inflammatory mediator linked with insulin resistance in obesity. Therapeutic effects of its antagonist on insulin resistance and diabetes were widely studied (101, 102). Bernstein et al. investigated effects of a TNF-α antagonist on inflammation in obese patients with metabolic syndrome by providing etanercept (103). Reduced concentrations of C-reactive protein, IL-6 and fibrinogen, and increased concentrations of adiponectin were observed. And they concluded that the TNF-α antagonist may attenuate inflammation by interrupting the inflammatory cascade that occurs in patients with abdominal obesity. Similarly, Stanley et al. (104) showed that the prolonged inhibition of TNF-α decreased
glucose level and increased high molecular weight adiponectin in obese subjects. However, CRP and IL-6 were unchanged after treatment.

Diet

In the last few decades, many bioactive compounds derived from food, such as EPA, DHA and resveratrol, etc. have been shown to have an anti-inflammatory effect. Some of those compounds are used as a dietary supplements and highly available in markets around the world.

The anti-inflammatory effects of long chain ω-3 polyunsaturated fatty acids (PUFA) have been well accepted. In vitro, ω-3 PUFA can inhibit the activation of NF-κB and decrease endothelial chemokines, MCP-1 and IL-8 expression in cultured endothelial cells (105). This is supported by a report from Weldon et al. in which eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) decreased NF-κB activation and induced an anti-inflammatory profile in lipopolysaccharide-stimulated human THP-1 macrophages. Cells pretreated with EPA and DHA decreased TNF-α, IL-1β and IL-6 production compared with control cells (106). Similar results were also seen in vivo studies. Xi et al. (107) fed mice with a diet contain either 20% corn oil or 17% of fish oil and 3% corn oil. They found that fish oil suppressed NF-κB activation and viral replication in the spleen and liver. In human studies, supplementation of ω-3 PUFA can reduce production of inflammatory cytokines (108, 109), prostaglandin E2 (110) and leukotriene B4 (109). The anti-inflammatory capacity of long chain ω-3 PUFA may have an important potential for reducing obesity-induced inflammation and improving insulin resistance.

Resveratrol is another bioactive compound found in food, including grapes and blueberries. Its anti-inflammatory effects also have been broadly studied. Gomez-zorita et al. reported that obese rats that received resveratrol for 6 weeks had lower serum concentrations of TNF-α, MCP-1 and CRP (111). IL-6 protein expression in epidermal adipose tissue was
reduced by 40%. The activity of NF-κB/p65 was also reduced. In another study, mice with obesity induced by a high fat diet were given resveratrol for 13 weeks (112). As a result, those mice showed decreased oxidative stress, reduced plasma concentrations of IL-6 and TNF-α and increased number of regulatory T cell in peripheral blood and spleen. The resveratrol supplement also inhibited macrophage infiltration in aortic tissue of db/db mice (113) which plays an important role in the development inflammation.

Summary

Obesity which is associated with a state of chronic inflammation has become a severe public health issue. Obesity-induced inflammation is considered the link between obesity and its related chronic diseases (4). Thus, it is important to find appropriate strategies to address the chronic inflammation during obesity.
Table 1-1. Inflammatory cytokines in obesity.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Distribution</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Increased cytokines</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>Secreted by macrophages and WAT adipocytes</td>
<td>Reduces insulin secretion and sensitivity. Stimulates lipolysis.</td>
</tr>
<tr>
<td>IL-6</td>
<td>Predominantly expressed by WAT, around 35%</td>
<td>Promotes glucose and lipid metabolism</td>
</tr>
<tr>
<td>IL-1</td>
<td>Secreted mainly by adipocytes and macrophages</td>
<td>Induces immune cells to infection site, thermogenesis</td>
</tr>
<tr>
<td>IL-7</td>
<td>Expressed by stromal and vascular endothelial cells</td>
<td>Stimulates the differentiation of hematopoietic stem cells</td>
</tr>
<tr>
<td>IL-8</td>
<td>Secreted by adipocytes and macrophages</td>
<td>Neutrophil chemotaxis</td>
</tr>
<tr>
<td>MCP1</td>
<td>Secreted by WAT</td>
<td>Reduces insulin sensitivity, increases macrophage infiltration during inflammation</td>
</tr>
<tr>
<td>MIP1-α</td>
<td>Secreted by macrophages</td>
<td>Recruits and activates polymorphonuclear leukocytes</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Decreased cytokines</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-10</td>
<td>Expressed by monocytes, macrophages, dendritic cells, B and T cells</td>
<td>Inhibits pro-inflammatory cytokines production</td>
</tr>
<tr>
<td>Omentin</td>
<td>Mainly secreted by WAT</td>
<td>Improves glucose uptake in human adipocytes</td>
</tr>
</tbody>
</table>

TNF-α: Tumor necrosis factor alpha; IL-6: interleukin 6; IL-1: interleukin 1; IL-7: interleukin 7; IL-8: interleukin 8; MCP-1: Monocyte chemotactic protein 1; MIP1-α: Macrophage inflammatory protein 1-α; IL-10: interleukin 10. Adapted from Makki k et al., 2013 (114).
Figure 1-1. Prevalence of obese in the United States. A: Prevalence of self-reported obesity among American adults by state and territory in 2016. There is no state that has a prevalence of obesity less than 20%. More than 35% of adults in Arkansas, Alabama, Mississippi, Louisiana and West Virginia were obese. Twenty states had a prevalence between 30% and 35%, and 22 states between 25% and 30%. Adjust from https://www.cdc.gov/obesity/data/prevalence-maps.html. * indicates Sample size <50 or the relative standard error (dividing the standard error by the prevalence) ≥ 30%.

B: Trends in obesity prevalence among adults aged 20 and over (age-adjusted) and youth aged 2–19 years: United States, 1999–2000 through 2015–2016 (1).

CHAPTER 2
INTRODUCTION AND RESEARCH OBJECTIVES

Background

As stated previously, obesity has become a serious health issue all over the world, especially in Western countries. In the United States, more than one-third of adults are obese (1). Obesity often comes with an increased risk of systemic inflammation that stems from the immune system (2, 3). When the immune system is chronically activated, a barrage of free radicals, oxidative products and inflammatory cytokines assault the human body, damaging cells and organs and increasing the risk for disease. If inflammation associated with obesity can be reduced, then fewer complications (e.g. diseases such as cardiovascular disease) might occur.

Oxidative stress is an important component of inflammation. It stimulates the activation of mediator signaling molecules such as NF-κB, which can up-regulate the production of inflammatory cytokines (5). GSH is central to redox defense in the body during oxidative stress, protecting host immune cells from free radicals. Daily consumption of dietary supplements that support GSH production could potentially be a method of counteracting the chronic inflammation associated with obesity. Two dietary supplements aged garlic extract (AGE) and GSH have been shown to replete GSH concentration in the body (115, 116). The potential subsequent effect of AGE and GSH on obesity-induced inflammation has not yet been studied yet.

Two randomized double-blind placebo-controlled clinical trials were conducted to determine if supplementation with AGE or GSH modulate immune function and reduce inflammation in obese adults. Also, cell culture studies were conducted to understand the mechanistic process.
Objectives and Hypotheses

The overall goal of my research was to determine if and how sulfur-containing dietary supplements, AGE and Setria® GSH, reduce chronic inflammation in adults with obesity. To achieve this goal, two randomized double-blind placebo-controlled human studies were conducted to determine these effects and an in-vitro study was carried out to investigate the mechanisms.

The specific aims are as follows:

**Specific aim 1**: To investigate if AGE supplementation will attenuate obesity-induced inflammation.

It is hypothesized that obese individuals consuming AGE supplement would show decreased pro-inflammatory and increased anti-inflammatory cytokine concentrations in serum and cell culture supernatant.

This aim was accomplished in three steps:

1) Characterize inflammation in the obese study participants.

2) Determine if AGE supplementation alters the distribution and activation of γδ-T cell and NK cells in obese participants.

3) Investigate if and how AGE changes the pattern of inflammatory cytokine expression and secretion in obese participants.

**Specific aim 2**: To investigate if consuming a GSH supplement will attenuate inflammation in older adults, with and without obesity

It is hypothesized that GSH supplementation would replenish GSH concentration, and reduce inflammation in older obese adults.

This aim was also accomplished in three steps:
1) Characterize GSH status in older adults, with and without obesity.

2) Determine if oral GSH supplementation alters the distribution and activation of γδ-T cell and NK cells in older adults with and without obesity.

3) Investigate if and how GSH changes the pattern of inflammatory cytokine synthesis and secretion in older adults with and without obesity.

Specific aim 3: To investigate the mechanism by which AGE and GSH exert their effects on obesity-induced inflammation.

It is hypothesized that the enhanced NF-κB and MAPK signaling pathways during obesity-induced inflammation would be reduced by AGE and GSH treatment.

Summary

Due to the essential role of GSH in alleviating oxidative stress, which plays an important role in obesity-induced inflammation, dietary supplements to support GSH production are a potential method for counteracting inflammation associated with obesity. It is hypothesized that AGE and Setria® GSH would reduce the chronic inflammation in obese adults through a mechanism involving NF-κB and MAPK signaling pathways.
CHAPTER 3
AGED GARLIC EXTRACT SUPPLEMENTATION MODIFIES INFLAMMATION AND IMMUNITY OF ADULTS WITH OBESITY: A RANDOMIZED, DOUBLE-BLIND, PLACEBO-CONTROLLED CLINICAL TRIAL

Introduction

Garlic has a long history of providing health benefits which are ascribed primarily to its organosulfur compounds. Those bioactive compounds in garlic can be classified into two categories, gamma-glutamylcysteines and cysteine sulfoxides (62). After absorbed intact, gamma-glutamylcysteines are hydrolyzed to S-allylcysteine and S-1-propenylcysteine (63), whereas cysteine sulfoxides are rapidly metabolized to allyl methyl sulfide, a volatile compound that accounts for the garlic odor (64).

AGE is a form of garlic manufactured through extraction and a proprietary process of aging. After harvesting, the garlic is stored in an aqueous ethanol solution for up to 20 months. This converts the organosulfur compounds in garlic into milder and less odiferous compounds, mainly S-allylcysteine.

Numerous studies have demonstrated the health benefits of AGE, such as neuroprotection, antihypertensive, and cholesterol-lowering effects (117-121). AGE is also able to modulate immune function. Patients with advanced cancer administered with AGE (4 capsules containing 500 mg AGE, 727 mg crystalline cellulose and 11 mg sucrose fatty acid ester) for 6 months showed an increased in both the number and activity of NK cells (122). Tabari et al. (123), administered AGE (100 mg/kg) to mice implanted with fibrosarcoma cells, intraperitoneally. As a result, mice that received AGE showed improved immune responses against their tumor. The immunity modulating function of AGE was also supported by a previous study (115). In that study, 120 healthy non-obese adults were randomized to consume AGE (2.56g) or a placebo, daily for 90 days. γδ-T and NK cells from participants who consumed
AGE showed greater proliferation after stimulation, than those of participants who consumed the placebo. In addition, NK cells of those consuming AGE had more NKG2D, a marker of cell activation, which plays an important role in NK cell cytotoxicity (124), expressed on their surface. In the same study, a decrease in the secretion of inflammatory cytokines from cultured peripheral blood mononuclear cells (PBMC) was observed. These findings suggest that AGE supplementation improved the natural killing capability of immune cells, while fewer inflammatory cytokines were being secreted. The purpose of this study was to extend the knowledge regarding AGE supplementation and its potential benefit of supporting immunity, by studying adults with obesity, many of whom also have concurrent systemic inflammation. The hypotheses of this study was that AGE supplementation would reduce obesity-associated chronic inflammation in healthy, adults with obesity through a modulation of their immune system. In order to examine this, the percentage of specific populations of lymphocytes in the total PBMC population were assessed, as well as concentrations of inflammatory cytokines in the serum, before and after the 6-week AGE intervention. The effect of daily AGE supplementation on blood lipid was also evaluated.

**Study Design and Methods**

**Participants and Study Design**

A total of 175 healthy adults with obesity who responded to public recruitment messages, were assessed for eligibility to participate in this double-blind, randomized, placebo-controlled clinical trial between July of 2014 and September of 2015. Of the 73 adults that met the inclusion criteria, 55 chose to enroll (Figure 3-1). All participant visits were conducted in the Clinical Nutrition Laboratory of the Food Science and Human Nutrition Building on University of Florida campus. Informed consent was obtained from all participants by trained personnel. This
study was approved by the Institutional Review Board at the University of Florida and registered at ClinicalTrials.gov with the identifier code NCT01959646.

The inclusion criteria for the study were: 25 - 65 years of age, WC >88 cm for women or >102 cm for men, BMI ≥ 30 kg/m², be willing to discontinue other dietary supplements, and not be taking any medication for cardiovascular disease. In addition, those with blood pressure greater than 130/85 mmHg, metabolic syndrome, diabetes, arthritis, severe allergies or other immune disorders were excluded from participating. Metabolic syndrome is defined as the presence of three or more of the following risk factors: WC >88 cm for women or >102 cm for men, blood pressure ≥130/85 mmHg, triglycerides ≥150 mg/dL, HDL cholesterol ≤40 mg/dL for men or ≤50 mg/dL for women, and fasting glucose ≥100 mg/dL (125).

**Sample Size Determination**

To determine the minimum number of participants needed to detect a difference between the AGE and placebo groups in the primary outcome, γδ-T cell numbers, and secondary outcomes, inflammatory markers, a power analysis was conducted. With an alpha level of 0.05 and a power of 0.80, based on previous data where the average percentage of γδ-T cells in peripheral blood of participants in the placebo group was 4.7% ± 2.7 and those in a previous intervention group averaged 9.9% ± 5.3, indicated that 15 individuals per group would be needed. To determine the number of participants needed per group to see a statistical reduction in CRP concentration from an average value of 3.0 mg/dL, an interquartile difference of 1.14 to 1.05 mg/dL was applied. Thirty-two participants were needed for each group.

**Study Procedures**

To determine eligibility, height, weight, blood pressure and a finger-prick blood sample were obtained. Eligible participants with similar BMI (difference of less than 3 kg/m²) were
matched as pairs and returned in the fasting state to the Clinical Nutrition Laboratory for a randomization visit. To ensure blinding of investigators and participants, capsules were supplied by Wakunaga of America Co., Ltd. (Mission Viejo, CA, USA), labeled as either A or B, and contained either AGE (0.6 g/capsule) or a placebo. A blind drawing was conducted by asking a participant to draw one out of two notes which were labeled as either A or B. If the participant was assigned to group A, the other of the pair would be assigned to group B. They were then given numbers from a randomization table provided by Wakunaga of America Co., Ltd. Both participants and investigators were blinded to the intervention assignments. Venous blood was drawn into heparinized vacuum tubes for PBMC isolation, and into serum collection tubes. Participants were given bottles of their assigned intervention, instructed to consume three capsules with food, twice a day for 6 weeks (3.6 g AGE/ day) and encouraged to continue their current dietary and exercise routines. After 3 weeks, participants returned bottles containing unused capsules and were given their second 3-week supply. After 6 weeks, participants again returned bottles containing unconsumed capsules for compliance assessment, underwent a second fasting blood draw, and completed a final questionnaire that asked about side effects experienced, consumption of other dietary supplements and medications, any significant changes in diet or exercise, and which treatment they thought they had consumed.

Anthropometric and Biochemical Measurements

Height, weight, blood pressure, WC and sagittal abdominal diameter (SAD) were measured in privacy by trained researchers at the beginning and end of the intervention period. All measurements were conducted in duplicate as follows: a. height, in centimeters, using a stadiometer; b. weight, in kilograms, using a calibrated floor scale; c. waist circumference, in centimeters, measured at the top of the ileac crest using a tape measure; d. sagittal abdominal
diameter, in centimeters, measured the external distance between the front of the abdomen and the small of the back at the iliac level line using an abdominal caliper.

Blood was drawn from fasting subjects into heparinized vacuum tubes for PBMC isolation, and serum collection tubes. Blood biochemical markers, including total cholesterol (TC), triglyceride (TG), low density lipoprotein cholesterol (LDL), high density lipoprotein cholesterol (HDL), non-high density lipoprotein cholesterol (non-HDL), and glucose (GLU), were determined using the Cholestech LDX® System (Alere™ Inc., Waltham, MA) which is an efficient and economical point of care testing for cholesterol and related lipids, and blood glucose. Universal precautions such as hand washing, glove change, new lancet, etc. were utilized for every finger prick throughout the assessment.

**Separation and Culture of PBMC**

To isolate PBMC, whole blood was layered on Lympholyte H® Cell Separation Media (Cedarlane Laboratories Ltd., Burlington, NC), as previously described (11). Isolated PBMC (1 \times 10^6) were cultured in wells of a 24-well plate in RPMI-1640 (Cellgro; Mediatech, Herndon, VA) complete medium (100,000 U/L penicillin; 100 mg/L streptomycin; 2 mmol/L L-glutamine; 25 mmol/L HEPES), containing 10% fetal bovine serum (FBS), IL-2 (1 ng/mL), IL-15 (1 ng/mL), and PHA-L (10 μg/mL), in a humidified, 5% CO₂ environment at 37°C. After 24 h, cell culture medium was collected for determination of inflammatory cytokine concentrations; cells were collected for measuring cell proliferation.

**Immune Cell Distribution within the Total PBMC Population**

Flow cytometry was used to detect different types of immune cells in freshly isolated PBMC. Cell surface markers were used to identify γδ-T cells (FITC-α human CD3, Alexa Fluor® 647-α human γδ-TCR), along with NK and NKT cells (FITC-α human CD3, PE-α human
CD56). All antibodies were obtained from eBioscience, San Diego, CA. Cells were incubated with antibodies at 4°C in the dark for 30 min, washed, centrifuged, fixed with 1% paraformaldehyde, and run in a BD Accuri™ C6 Plus flow cytometer (Becton Dickinson, San Jose, CA) within 48 h. Data were analyzed using FlowJo (version 10.1) analysis software (FlowJo, LLC., Ashland, OR). Results are presented as a percentage of the total lymphocyte population.

Total GSH and C-reactive Protein Concentrations Determination

Total intracellular GSH was measured in 1 x 10^7 PBMC using a commercial GSH assay kit (Cayman, Ann Arbor, MI) according to kit directions.

The CRP concentration in the serum was determined by ELISA using a human C-Reactive Protein/CRP Quantikine® ELISA Kit (R&D System Inc., Minneapolis, MN) according to the manufacturer’s instructions.

Inflammatory Cytokine Determination

Concentrations of IL-6, TNF-α, leptin and adiponectin in the serum and IL-6, TNF-α, MIP-1α and IL-10 in supernatant cell culture medium were determined using a human cytokine multiplex immunoassay kit (Milliplex® Map Kit, EMD Millipore Corp., Billerica, MA). The assays were performed following manufacturer directions.

Statistical Analysis

All statistical analyses were performed using SAS JMP, v10 (SAS Institute, Cary, NC). Descriptive statistics were used to show the demographic and anthropometric characteristics of participants. Independent t-test was performed to compare the differences between the two groups at baseline. A one-way analysis of covariance (ANCOVA) was conducted to analyze the differences in all parameters between the two groups at the end of the study, while controlling
for respective baseline values. Categorical data were analyzed by using Chi-squared test. There were three participants with compliance of less than 80%. Both, intent to treat analysis and per-protocol analysis were performed. Significant differences were observed when those three participants were excluded from the analysis, but not intent to treat analysis. Thus, data collected from those participants were not included. Two additional subjects reported illnesses at the post-intervention blood draw. Statistical analyses were performed both with and without those two participants and as no differences were observed, those data were included in all analyses. All data are presented as the mean ± SEM, with p < 0.05 considered significant.

Results

Baseline Demographic Characteristics

Subject demographics were examined for differences between those randomized to consume AGE or the placebo and found that the groups were comparable in age, gender distribution, anthropometric parameters and blood biochemical parameters, except for baseline TG concentration (Table 3-1). After 6 weeks of intervention, no changes were observed in blood pressure, body weight and other anthropometric measures. Self-reported side effects, illnesses and related behaviors of participants throughout the study are found in Table 3-2. Although 1/3 participants from AGE group reported having side effects, all these participants still consumed the capsules as required. The primary symptom was slight GI irritation.

Intervention Compliance

Based on pill counts, average compliance levels were 93.4% in the placebo group and 92.2% in the AGE group (Figure 3-2 A). Cellular GSH concentrations were determined as an indirect measure for compliance. Although changes in total PBMC GSH concentrations from
baseline were not significantly different (p = 0.06), the concentration tended to increase in the AGE group and decrease in the placebo group (Figure 3-2 B).

**Immune Cell Distribution**

Table 3-3 shows the proportions of γδ-T, NKT and NK cells in the total PBMC population. At the end of the intervention period, the percentage of γδ-T cells in AGE group (p = 0.03) was significantly higher, while the percentage of NKT cells was lower (p = 0.02) when compared with the placebo group, while controlling for respective baseline measures. The percentage of NK cells remained the same.

**Markers of Inflammation**

Several serum markers of inflammation were examined before and after the intervention (Table 3-4). In response to the intervention, significant differences were seen in IL-6 and TNF-α concentrations between the two groups. After the intervention, participants consuming AGE had lower serum IL-6 (p = 0.04) concentration and lower serum TNF-α concentration (p = 0.05), controlling for respective baseline values.

No significant differences in CRP, leptin, and adiponectin were observed between the two groups in response to the intervention. The average concentration of CRP in the serum before the intervention was higher than normal in both the placebo (5.7 mg/dL) and AGE (4.4 mg/dL) groups. After the intervention, the concentration of CRP was very similar to baseline. No significant differences were seen at either time point between the two groups (Figure 3-4 C). The mean leptin concentration increased by 2.1 ng/mL in the placebo and by 0.8 ng/mL in the AGE group. These changes were not significantly different between the two groups (Figure 3-4 D). Adiponectin concentrations decreased in both groups, the placebo by 687.4 ng/mL and AGE by
647.9 ng/mL, but the decreases were not statistically different between the two groups (Figure 3-4 E).

Concentrations of cytokines (IL-6, TNF-α, MIP-1α and IL-10) secreted by stimulated PBMC cultured for 24 h were determined pre- and post-intervention. No differences from baseline values were observed in either group at either time point (Table 3-5).

**Clinical Parameters**

After six week of supplementation, blood LDL concentrations in response to the intervention differed between the AGE and placebo groups (p = 0.05), controlling for baseline LDL concentrations. In the AGE group, the average LDL concentration was 118.0 ± 28.4 which was higher than that in the placebo group (107.7 ± 31.3). While there was an observed decrease of both TC and non-HDL in the AGE group, differences between the AGE and control groups were not significant for either parameter (TC, p = 0.07; non-HDL p = 0.08). Although TG concentrations were different at baseline between the two groups, there were not differences in change in TG concentration between the two groups during the intervention period (p = 0.18). There were also no significant differences in the changes of HDL and GLU concentrations between the two groups (Table 3-6).

**Discussion**

In this 6-week, double-blind, randomized, placebo-controlled clinical study, the effects of AGE supplementation on immune cells and markers of inflammation were assessed. In adults with obesity, 6 weeks of AGE supplementation was able to modulate immune cell distribution and decrease serum concentrations of IL-6 and TNF-α, factors that play important roles in the development of obesity-induced inflammation. Moreover, blood LDL concentration, which often
increases with obesity and is a major health concern (126), was significantly lower after AGE supplementation.

AGE and Inflammatory Mediators During Obesity-induced Inflammation

Adipocytes, the major cell type present in adipose tissue, can secrete IL-6, leptin, and adiponectin. In obesity, adipocytes tend to increase in both number and size, which results in excess adipokine secretion and a pro-inflammatory environment in the adipose tissue. It is well established that concentrations of inflammatory mediators in the serum increase in obesity, and are positively correlated with BMI and WC (16, 23, 127). Confirming those findings, serum CRP, IL-6, TNF-α and leptin concentrations in the current study were all elevated at baseline, an indication of inflammation in the obese participants. After the intervention, those consuming AGE had lower serum IL-6 and TNF-α concentrations after the six week intervention, suggesting that AGE supplementation might help to prevent the progress of inflammation. No changes in serum CRP or leptin concentrations were observed. Very few human studies have assessed the effect that consumption of AGE or garlic-associated compounds might have on CRP. A study conducted by Zeb et al. (128) showed that the consumption of a capsule containing AGE and Coenzyme Q10 for 12 months significantly decreased CRP concentration compared with a placebo. Since the capsule contained both AGE and Coenzyme Q10, the authors were unable to attribute the anti-inflammatory effect they saw to either one of the compounds. In addition, the participants in that study were all male firefighters, a very distinct population. In another study, garlic powder (2.1 g/d) was consumed by overweight participants for three months and, as what was found in this study, there were no significant differences in serum CRP concentrations between the groups after the intervention (129). The effect of AGE consumption on leptin has only been studied in animals thus far. Perez-Torres et al. showed that rats with metabolic
syndrome had elevated serum leptin concentrations and that AGE returned them to control concentrations (130). However, this benefit was not seen in this study. It may be due to the differences in metabolism between humans and rats.

In the obese, concentrations of anti-inflammatory mediators are reduced and are negatively correlated with BMI (131). One of those anti-inflammatory mediators, adiponectin, is secreted by adipose tissue. Ryo et al. (132) showed that adiponectin concentration is negatively associated with the clinical phenotype of metabolic syndrome. In a study conducted by Gómez-Arbeláez et al. (133), AGE supplementation improved adiponectin concentration in obese adults with metabolic syndrome. In this study, where having metabolic syndrome was exclusionary, no change in serum adiponectin concentration was observed in either group after the 6-week intervention. All participants in this study had relatively high concentrations of adiponectin prior to the intervention, with average baseline concentrations of 14.2 μg/mL, more than double the 5.9 μg/mL in the Gómez-Arbeláez study. The differences in metabolic health and initial adiponectin concentrations of participants in the current study and in that of Gomez-Arbelaez, may explain the variation in the adiponectin response to AGE.

Cytokines secreted by cultured PBMCs were also assessed, but found not to differ between treatments. The reason is not clear, however variability among participants was apparent.

**AGE and Immune Cell Populations During Obesity-induced Inflammation**

Similar to changes in inflammatory mediators, alterations in immune cell population numbers were also observed. Obesity has a detrimental impact on γδ-T cell populations because of the low-grade chronic inflammation. Human obesity is associated with a reduction of γδ-T cells in the peripheral blood, and the number is negatively correlated to the severity of the
obesity (134). In contrast, results from an animal study showed an increase in the number of γδ-T cells in adipose tissue (36). The decrease in the number of γδ-T cells in peripheral blood may be due to their infiltration into adipose tissue. In fact, a low baseline percentage of γδ-T cells was observed in the blood of the healthy obese adults enrolled in this study. While γδ-T cells increase in adipose tissue, NKT cells are diminished from it (135). CD3+CD56+ NKT cells are highly enriched in liver and adipose tissue, but rare in peripheral blood, around 2%. At the start of intervention, NKT cells comprised a much higher high proportion (6.7%) of that lymphocyte population, perhaps as a result of the migration of those cells from adipose tissue into the circulation. After supplementation with AGE, the increase in the percentage of γδ-T cells and the inhibition of increase in the percentage of NKT cells in the circulating blood of participants indicated that AGE supplementation had modulated their immunity. Since the participants in our study were healthy obese adults, no changes in NK cell numbers were observed, consistent with results from previous studies (31, 32).

**AGE and Risk Factors of Cardiovascular Diseases**

The cardiovascular benefits of AGE supplementation, including the anti-hypertensive and cholesterol-lowering effects, have been illustrated in numerous studies. Ried et al. demonstrated that AGE was effective in lowering blood pressure in patients with uncontrolled hypertension (117). In that study, participants were asked to consume 0.96 g/day of AGE for 12 weeks, or a placebo, and blood pressure was measured at baseline, 4, 8 and 12 weeks. Participants with uncontrolled hypertension in the AGE group experienced a decrease in systolic blood pressure throughout the 12-week intervention period. In this study, all participants started with blood pressure levels of less than 130/85 mmHg, which perhaps explains why no anti-hypertensive effects were observed.
As early as 1996, Steiner et al. showed that daily consumption of 7.2 g of AGE for 6 months had beneficial effects on the lipid profile of moderately hypercholesterolemic participants, with decreases in TC (7.0%) and LDL (4.6%) concentrations (136). Yeh et al. demonstrated a cholesterol-lowering effect of AGE using both human and animal studies (121). In the human study, 36 hypercholesterolemic participants, with an average BMI of 25.5 kg/m², were asked to consume 7.2 g of AGE per day for 5 months. After the intervention, the concentration of TC had decreased by 7% and the concentration of LDL cholesterol had decreased by 10%. Similar results were observed in their animal study, where AGE supplementation lowered total cholesterol concentration by 15%. LDL concentration in participants that consumed AGE decreased by 5.2% in this study. There was also a trend for TC and non-HDL concentrations to decrease, indicating that AGE did improve the lipid profile of adults with obesity. Although the LDL decrease that was observed in this study was not as pronounced as that of earlier studies, this could be due to the shorter intervention period.

Strength and Limitations

This study has several strengths. To our knowledge, this is the first report that evaluates the effect of AGE supplementation on immune cell populations in adults with obesity-induced inflammation. As obesity affects more than 600 million adults worldwide (137), identifying successful interventions to moderate the negative effects of obesity, like systemic inflammation and associated chronic diseases such as CVD, are imperative. Here, not only the response of immune cell populations was investigated, but also inflammatory mediators. Participants were asked to maintain their usual diet and activity patterns, so body weight remained unchanged throughout the intervention period and was not a confounding factor. A limitation of interpreting the findings from this and previous work regarding AGE supplementation is the variance in AGE
dose and participant characteristics. Participants in the intervention group of this study consumed 3.6 g/day of AGE for 6 weeks and were obese, without metabolic syndrome. Other investigations discussed here provided doses ranging from 0.96 g/day to adults with uncontrolled hypertension to 7.2 g/day for adults with hypercholesterolemia. Furthermore, the length of the intervention periods differs significantly among the studies. As the intervention lasted only 6 weeks in this study, it is hard to evaluate the long-term effects of AGE supplementation on inflammation and immune function in adults with obesity, . The other studies had AGE intervention periods that ranged from 12 weeks to 6 months. Given these limitations, additional immunity studies with longer intervention periods are warranted to determine the long-term benefits of AGE consumption on the immune health of adults with obesity.

**Summary**

In summary, the changes in immune cells and inflammatory mediators show that the consumption of AGE can produce an anti-inflammatory effect in adults with obesity. However, the mechanism by which AGE exerts its effect on obesity-induced inflammation is still not clear. One potential mechanism may involve hydrogen sulfide (H$_2$S), a recently discovered second messenger shown to prevent the activation of the NF-κB signaling pathway, which consequently attenuates the production of pro-inflammatory cytokines (138, 139). The organosulfur compounds in aged garlic extract, such as SAC, have been suggested as H$_2$S mediators by increasing its endogenous production (140, 141), which may drive the suppression of obesity induced-inflammation. Future studies focusing on determining the mechanism by which AGE affects obesity-induced inflammation were suggested, to increase the understanding of the potential health benefits of AGE consumption.
Table 3-1. Demographics and baseline clinical characteristics of study participants at baseline.

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Placebo</th>
<th>AGE</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Demographics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>48</td>
<td>24</td>
<td>24</td>
<td>N/A</td>
</tr>
<tr>
<td>Age (y)</td>
<td>42.4 ± 2.1</td>
<td>45.7 ± 2.5</td>
<td>45.9 ± 2.4</td>
<td>0.33</td>
</tr>
<tr>
<td>Gender (n, %)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>15 (31.3%)</td>
<td>8 (33.3%)</td>
<td>7 (29.2%)</td>
<td>0.76</td>
</tr>
<tr>
<td>Female</td>
<td>33 (68.7%)</td>
<td>17 (66.7%)</td>
<td>16 (70.8%)</td>
<td></td>
</tr>
<tr>
<td><strong>Blood Pressure</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>121 ± 2</td>
<td>122 ± 2</td>
<td>121 ± 2</td>
<td>0.91</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>84 ± 2</td>
<td>86 ± 3</td>
<td>81 ± 2</td>
<td>0.17</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>104.0 ± 2.8</td>
<td>104.1 ± 3.5</td>
<td>103.8 ± 4.3</td>
<td>0.94</td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>36.4 ± 0.9</td>
<td>36.3 ± 1.2</td>
<td>36.5 ± 1.3</td>
<td>0.90</td>
</tr>
<tr>
<td>WC (cm)</td>
<td>113.6 ± 1.9</td>
<td>114.0 ± 2.7</td>
<td>113.2 ± 2.8</td>
<td>0.85</td>
</tr>
<tr>
<td>SAD (cm)</td>
<td>25.4 ± 0.5</td>
<td>25.4 ± 0.5</td>
<td>25.4 ± 0.9</td>
<td>0.99</td>
</tr>
<tr>
<td><strong>Clinical</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TC (mg/dL)</td>
<td>185.4 ± 4.9</td>
<td>188.1 ± 6.6</td>
<td>183.4 ± 7.7</td>
<td>0.59</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>103.5 ± 5.2</td>
<td>114.0 ± 7.5</td>
<td>91.6 ± 6.2</td>
<td>0.03</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>54.3 ± 2.4</td>
<td>51.9 ± 3.1</td>
<td>55.8 ± 3.4</td>
<td>0.42</td>
</tr>
<tr>
<td>Non-HDL (mg/dL)</td>
<td>131.2 ± 4.7</td>
<td>135.9 ± 6.1</td>
<td>127.6 ± 7.2</td>
<td>0.31</td>
</tr>
<tr>
<td>LDL (mg/dL)</td>
<td>113.4 ± 4.5</td>
<td>113.1 ± 6.0</td>
<td>113.9 ± 7.1</td>
<td>0.93</td>
</tr>
<tr>
<td>GLU (mg/dL)</td>
<td>97.8 ± 3.0</td>
<td>95.9 ± 2.0</td>
<td>99.7 ± 5.7</td>
<td>0.53</td>
</tr>
<tr>
<td>CRP (mg/dL)</td>
<td>5.1 ± 0.6</td>
<td>4.4 ± 0.8</td>
<td>5.7 ± 0.9</td>
<td>0.28</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM. Independent T test was performed to compare the differences between the two groups at baseline. SBP: systolic blood pressure; DBP: diastolic blood pressure; BMI: body mass index; WC: waist circumference; SAD: sagittal abdominal diameter; TC: total cholesterol; HDL: high density lipoprotein cholesterol; LDL: low density lipoprotein cholesterol; non-HDL: non-high density lipoprotein cholesterol; GLU: glucose; CRP: C-reactive protein.
Table 3-2. Other characteristics of participants.

<table>
<thead>
<tr>
<th>Questions</th>
<th>Placebo (n=26)</th>
<th>AGE (n=24)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>During the study, did you experience any side effect(s) ?</td>
<td></td>
<td></td>
<td>0.06</td>
</tr>
<tr>
<td>Yes</td>
<td>3 (11.5%)</td>
<td>8 (33.3%)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>23 (88.5%)</td>
<td>16 (66.7%)</td>
<td></td>
</tr>
<tr>
<td>During the study, did you take a medication, any dietary supplement?</td>
<td></td>
<td></td>
<td>0.73</td>
</tr>
<tr>
<td>Yes</td>
<td>11 (42.3%)</td>
<td>9 (37.5%)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>15 (57.7)</td>
<td>15 (62.5%)</td>
<td></td>
</tr>
<tr>
<td>During the study did your diet or exercise habits change?</td>
<td></td>
<td></td>
<td>0.44</td>
</tr>
<tr>
<td>Yes</td>
<td>4 (15.4%)</td>
<td>2 (8.3%)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>22 (84.6%)</td>
<td>22 (91.7%)</td>
<td></td>
</tr>
<tr>
<td>Were you sick during either one of the / blood draws?</td>
<td></td>
<td></td>
<td>0.60</td>
</tr>
<tr>
<td>Yes</td>
<td>2 (7.7%)</td>
<td>1 (4.2%)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>24 (92.3%)</td>
<td>23 (95.8%)</td>
<td></td>
</tr>
<tr>
<td>Do you have allergies?</td>
<td></td>
<td></td>
<td>0.67</td>
</tr>
<tr>
<td>Yes</td>
<td>7 (26.9%)</td>
<td>5 (21.8%)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>19 (73.1%)</td>
<td>18 (78.2%)</td>
<td></td>
</tr>
<tr>
<td>Did you have a flu shot this season?</td>
<td></td>
<td></td>
<td>0.92</td>
</tr>
<tr>
<td>Yes</td>
<td>9 (34.6%)</td>
<td>8 (33.3%)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>17 (65.4%)</td>
<td>16 (66.7%)</td>
<td></td>
</tr>
<tr>
<td>Hand washing and hygiene practices</td>
<td></td>
<td></td>
<td>0.16</td>
</tr>
<tr>
<td>Excellent</td>
<td>13 (50.0%)</td>
<td>7 (29.2%)</td>
<td></td>
</tr>
<tr>
<td>Very good</td>
<td>11 (42.3%)</td>
<td>11 (45.8%)</td>
<td></td>
</tr>
<tr>
<td>Good</td>
<td>2 (7.7%)</td>
<td>6 (25.0%)</td>
<td></td>
</tr>
</tbody>
</table>

One participant didn't complete the final questionnaire. A chi-squared test was performed to compare differences between the two groups.
Table 3-3. Immune cell distribution at baseline and completion of the study

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th></th>
<th>AGE</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Week 6</td>
<td>Baseline</td>
<td>Week 6</td>
</tr>
<tr>
<td>γδ T cell %</td>
<td>1.7 ± 0.2</td>
<td>1.6 ± 0.2</td>
<td>1.2 ± 0.2</td>
<td>1.8 ± 0.2*</td>
</tr>
<tr>
<td>NKT%</td>
<td>7.5 ± 1.1</td>
<td>8.7 ± 1.3</td>
<td>6.5 ± 1.2</td>
<td>6.5 ± 1.1*</td>
</tr>
<tr>
<td>NK%</td>
<td>14.1 ± 1.2</td>
<td>15.1 ± 1.4</td>
<td>13.3 ± 1.2</td>
<td>12.6 ± 1.1</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± SD. A one-way ANCOVA was performed to compared proportion of γδ T cell, NKT cell and NK cell in the total PBMC between the two groups on controlling for respective baseline measures. NK: natural killer.

* Indicates statistically significant differences between the two groups at Week 6 on controlling for respective baseline values, p<0.05.
Table 3-4. Serum biomarkers at baseline and completion of the study

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th></th>
<th>AGE</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Week 6</td>
<td>Baseline</td>
<td>Week 6</td>
</tr>
<tr>
<td>CRP (mg/dL)</td>
<td>4.4 ± 0.8</td>
<td>6.2 ± 1.4</td>
<td>5.7 ± 0.9</td>
<td>6.5 ± 1.1</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>3.0 ± 0.5</td>
<td>3.8 ± 0.9</td>
<td>2.8 ± 0.5</td>
<td>2.8 ± 0.5*</td>
</tr>
<tr>
<td>TNF-a (pg/mL)</td>
<td>3.9 ± 0.4</td>
<td>4.2 ± 0.4</td>
<td>3.9 ± 0.4</td>
<td>3.9 ± 0.3*</td>
</tr>
<tr>
<td>Leptin (ng/mL)</td>
<td>27.5 ± 2.3</td>
<td>29.6 ± 2.4</td>
<td>26.1 ± 2.7</td>
<td>26.9 ± 3.1</td>
</tr>
<tr>
<td>Adiponectin (μg/mL)</td>
<td>14.2 ± 2.5</td>
<td>13.5 ± 1.4</td>
<td>13.7 ± 1.7</td>
<td>13.1 ± 1.7</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± SD. A one-way ANCOVA was performed to compared all inflammatory mediators between the two groups on controlling for respective baseline measures. CRP: C-reactive protein; IL-6: interleukin 6; TNF-a: tumor necrosis factor alpha. * Indicates statistically significant differences between the two groups at Week 6 on controlling for respective baseline values, p<0.05.
Table 3-5. Cytokine secreted by PBMC after 24 h stimulation of PHA-L

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th></th>
<th>AGE</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Week 6</td>
<td>Baseline</td>
<td>Week 6</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>5799.3±84.3</td>
<td>6971.4±276.8</td>
<td>6082.7±229.6</td>
<td>6687.5±283.2</td>
</tr>
<tr>
<td>IL-10 (pg/mL)</td>
<td>761.2±99.8</td>
<td>1452.9±207.5</td>
<td>804.5±110.0</td>
<td>868.6±89.0</td>
</tr>
<tr>
<td>MIP-1α (pg/mL)</td>
<td>6544.5±67.7</td>
<td>6899.7±133.0</td>
<td>6631.2±128.5</td>
<td>6653.4±184.1</td>
</tr>
<tr>
<td>TNF-α (μg/mL)</td>
<td>1550.2±214.8</td>
<td>2416.8±334.7</td>
<td>1390.8±192.3</td>
<td>1869.0±255.8</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± SD. A one-way ANCOVA was performed to compared differences between the two groups on controlling for respective baseline measures. IL-6: interleukin 6; IL-10: interleukin 10; MIP-1α: macrophage inflammatory protein 1 alpha; TNF-α: tumor necrosis factor alpha.
Table 3-6. Blood lipids and glucose of participants at the baseline and completion of the study

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>AGE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Week 6</td>
</tr>
<tr>
<td><strong>TC (mg/dL)</strong></td>
<td>188.1 ± 32.2</td>
<td>191.4 ± 35.2</td>
</tr>
<tr>
<td><strong>TG (mg/dL)</strong></td>
<td>114.0 ± 36.6</td>
<td>104.4 ± 34.2</td>
</tr>
<tr>
<td><strong>HDL (mg/dL)</strong></td>
<td>52.3 ± 6.4</td>
<td>52.4 ± 14.3</td>
</tr>
<tr>
<td><strong>non-HDL (mg/dL)</strong></td>
<td>135.9 ± 30.0</td>
<td>138.8 ± 6.0</td>
</tr>
<tr>
<td><strong>LDL (mg/dL)</strong></td>
<td>113.1 ± 29.3</td>
<td>118.0 ± 28.4</td>
</tr>
<tr>
<td><strong>GLU (mg/dL)</strong></td>
<td>113.1 ± 29.3</td>
<td>118.0 ± 28.4</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± SD. A one-way ANCOVA was performed to compared clinical characteristics between the two groups on controlling for respective baseline measures. TC: total cholesterol; TG: triglycerides; HDL: high density lipoprotein cholesterol; non-HDL: non-high density lipoprotein cholesterol; LDL: low density lipoprotein cholesterol; GLU: glucose. ^* Indicates statistically significant differences between the two groups at Week 6 on controlling for respective baseline values, p<0.05.
Figure 3-1. Overall flow of participants assessed for eligibility, enrolled in the AGE study, randomized to different treatments and analyzed statistically.
Figure 3-2. Participant compliance. A: Percentage of aged garlic extract (AGE) and placebo capsules consumed by participants during the 6-week study. B: Change from baseline of GSH concentrations in $1 \times 10^7$ peripheral blood mononuclear cells (PBMC). Intracellular GSH tended to increase in AGE group, but did not reach statistical significance.
CHAPTER 4
ORAL SUPPLEMENTATION WITH 500 mg GSH DOES NOT MODIFY OBESITY-INDUCED INFLAMMATION IN OLDER ADULTS: A RANDOMIZED, DOUBLE-BLIND, PLACEBO-CONTROLLED CLINICAL TRIAL

Introduction

GSH is a tripeptide, which can eliminate ROS through directly reacting or the reaction is catalyzed by three members of GSH peroxidase family and peroxiredoxin 6 (142).

Intracellular GSH is reduced in aged individuals (143). The most important consequence of a low GSH concentration is compromised immune function. The human immune system requires GSH for two reasons (144). Since the immune system produces free radicals to kill pathogens, GSH can protect host immune cells with its antioxidant mechanism. Lymphocytes, cells of the immune system, are dependent on adequate GSH level; T-cell proliferation and cytotoxic NK cell activity are functions that are impaired under low GSH conditions (145, 146). On the other hand, all forms of GSH are also diminished in individuals with obesity (6), which might link to the development of obesity-induced inflammation, leading to obesity associated chronic diseases. ROS plays an important role in the development of inflammation through activating NF-κB and MAPK activation (5, 147), which can up-regulate the production of inflammatory cytokines. Without enough GSH to eliminate extra ROS produced in the body, those pathways are chronically activated and constantly promote secretion of inflammatory cytokines and chemokines.

Thus, older adults with obesity are at higher risk of obesity related complications. If the GSH concentration in this population can be replenished, the obesity-induced inflammation may be reduced. The first step, replenishment of GSH with oral supplementation, was demonstrated successfully in a randomized controlled trial conducted by Richie et al. (116). Here, oral supplementation with GSH can increase GSH concentration in plasma, red blood cell and
lymphocytes. The purpose of this randomized double-blind placebo-controlled study is to test the hypothesis that consuming a GSH supplementation for 4 months will improve immune function and decrease the low-grade inflammation in older adults with obesity. The supplement used in this study is Setria® GSH, which is a highly absorbable form of L-GSH supplement produced through using a proprietary fermentation process.

**Study Design and Methods**

**Study Design and Participants**

This is a secondary analysis of participants that were enrolled in the GSH study (Oral GSH Supplementation in Older Healthy Adults and its Effect on the Number of Healthy Days Experienced during Four Months of Supplementation Compared to Placebo). A total of 315 healthy older adults were assessed for eligibility to participate in this double-blind, randomized, placebo-controlled clinical trial between December of 2015 and June of 2016. Of the 124 adults that met the inclusion criteria, 114 agreed to participate and received allocation (Figure 4-1). All participant visits were conducted in the Clinical Nutrition Laboratory of the Food Science and Human Nutrition Building on University of Florida campus. This study was approved by the Institutional Review Board at the University of Florida.

The inclusion criteria for the study were: 50 - 75 years of age, had at least one cold in the past year, be willing to discontinue using dietary supplements and probiotics. Individuals with a compromised immune system, including diabetes, arthritis, HIV/AIDS, severe allergies, hepatitis, cancer and autoimmunity were excluded.

**Sample Size Determination**

To determine the minimum number of participants needed to detect a difference between the GSH and placebo groups in primary outcome, healthy days experienced during the flu
season, a power analysis was conducted. Based on an expected difference of 0.7 healthy days experienced during the flu season, 56 individuals per group would be needed with an alpha level of 0.05 and a power of 0.80.

**Study Procedure**

Eligible participants were scheduled a visit to the Clinical Nutrition Laboratory to obtain their consent for participation, undergo anthropometric and blood pressure measurements and complete two dietary screener questionnaires. Then, participants were asked to return for a blood draw and receive their randomization by drawing a card labeled with “A” or “B” from an opaque envelope. Venous blood was drawn into heparinized vacuum tubes for PBMC isolation, and into serum collection tubes. Participants were then given bottles of their assigned intervention, instructed to consume 2 capsules with food every morning for 4 months (500mg/day) and encouraged to continue their current dietary and exercise routines. To ensure blinding of investigators and participants, capsules were supplied by Kyowa Hakko Bio Co., Ltd. (Tokyo, Japan), labeled as either A or B, and contained either Setria® GSH (250mg/capsule) or a placebo. After 2 months, participants returned bottles containing unused capsules and were given their second 2-month supply. Participants were also asked to complete a one page questionnaire. After 4 months, participants again returned bottles containing unconsumed capsules for compliance assessment, underwent a second fasting blood draw, and completed two dietary screener questionnaires and a final questionnaire that asked about side effects experienced, consumption of other dietary supplements and medications, any significant changes in diet or exercise, and which treatment they thought they had consumed.
Anthropometric and Biochemical Measurements

Height, weight, blood pressure, WC and SAD were measured in privacy by trained researchers at the beginning and end of the intervention period. All measurements were taken in duplicate as described previously. Blood was drawn from fasting subjects into heparinized vacuum tubes for PBMC isolation, and serum collection tubes. Blood biochemical markers, including TC, TG, LDL, HDL, non-HDL, and GLU, were determined using the Cholestech LDX® System (Alere™ Inc., Waltham, MA). Universal precautions such as hand washing, glove change, and new lancet, etc. were utilized for every finger prick throughout the assessment.

Separation and Culture of PBMC

To isolate PBMC, whole blood was layered on Lympholyte H® Cell Separation Media (Cedarlane Laboratories Ltd., Burlington, NC, USA), as previously described (10). Isolated PBMC (1 × 10⁶) were cultured in wells of 24-well plates in RPMI-1640 (Cellgro; Mediatech, Herndon, VA, USA) complete medium (100 U/mL penicillin; 100 µg/mL streptomycin; 2 mM L-glutamine; 25mM HEPES), containing 10% autologous serum, IL-2 (1 ng/mL), IL-15 (1 ng/mL), β-Mercaptoethanol (50 μM) and PHA-L (10 μg/mL), in a humidified, 5% CO₂ environment at 37°C. After 24 h, cell culture medium was collected for determination of inflammatory cytokine concentrations.

Immune Cell Distribution within the Total PBMC Population

Flow cytometry was used to detect different types of immune cells in freshly isolated PBMC. Cell surface markers were used to identify γδ-T cells (PE Cy 7-α human CD3, PE-α human γδ-TCR), and NK and NKT cells (PE Cy 7-α human CD3, APC-α human CD56). All antibodies were obtained from eBioscience, San Diego, CA, USA. Cells were incubated with
antibodies at 4°C in the dark for 30 min, washed, centrifuged, fixed with 1% paraformaldehyde, and run through a BD Accuri™ C6 Plus flow cytometer (Becton Dickinson, San Jose, CA, USA) within 48 hours. Data were analyzed using FlowJo (version 10.1) analysis software (FlowJo, LLC., Ashland, OR, USA). Results are presented as a percentage of the total lymphocyte population.

**Total GSH**

Total intracellular GSH was measured in 1 x 10^7 PBMC using a commercial GSH assay kit (Cayman, Ann Arbor, MI, USA) following kit directions.

**Inflammatory Cytokine Determination**

IL-6, TNF-α, MIP-1α and IL-10 in supernatant cell culture medium were determined using human cytokine multiplex immunoassay kits (Milliplex® Map Kit, EMD Millipore Corp., Billerica, MA, USA). The assays were performed following manufacturer’s directions.

**Statistical Analysis**

All statistical analyses were performed using SAS JMP, v10 (SAS Institute, Cary, NC, USA). For this analysis, participants with obesity were separated from those without. Thus, all participants were divided into four groups, non-obese control group (NC), obese control group (OC), non-obese GSH group (NG) and obese GSH group (OG). Descriptive statistics were used to show the demographic and anthropometric characteristics of participants. One-way analysis of variance (ANOVA) and Student-Newman-Keuls multiple comparison post-hoc tests were used to analyze the differences of changes in all parameters from baseline, calculated as post- minus pre-intervention, among the four groups. There were four participants with compliance of less than 80%. Statistical analyses were performed both with and without those four data sets and, as
no differences were observed, those data were included in all analyses. All data are presented as the mean ± SEM, with p < 0.05 considered significant.

**Results**

**Baseline Participant Demographic Characteristics**

Participant demographic characteristics were examined for differences among the four groups. NC and NG, OC and OG groups were comparable in age, gender distribution, anthropometric parameters and blood biochemical parameters, except for baseline TG concentration, respectively (Table 4-1). Each group had reported similar rate of side effects, allergies, changes in exercise and diet and flu shot, etc., during the intervention period (Table 4-2).

**Compliance**

Based on pill counts, average compliance levels were 98.1% in NC group, 95% in OC group, 95.2% in NG group and 94.5% in OG group. (Figure 4-2A). Intracellular GSH concentrations in PBMC was determined as an indirect measure for compliance. However, not significant differences were observed among the four groups after the 4-month intervention, or within each group at different time point (Fig 4-2 B).

**Immune Cell Distribution**

The average percentages of γδ-T, NKT and NK cells in the total PBMC population before and after the intervention among the four groups were not different (Figure 4-3).

**Respiratory Burst**

A respiratory burst assay was run to determine the functional response of neutrophils. The stimulation index (ratio of reactive oxidants released by neutrophils with PMA stimulation to that without PMA stimulation) was not different (Figure 4-4).
Markers of Inflammation

Concentrations of cytokines (IL-6, TNF-α, MIP-1α and IL-10) secreted by stimulated PBMC cultured for 24 h were determined pre- and post-intervention. No differences from baseline values were observed in either group (Figure 4-5).

Clinical Parameters

As expected, participants with obesity showed a high blood TG and GLU concentration and low blood HDL concentration at baseline. However, those clinical parameters stayed the same after the 4-month intervention (figure 4-6).

Discussion

In this 4-month, double-blind, randomized, placebo-controlled clinical study, the effects of GSH supplementation on immune cells and markers of inflammation in older adults with or without obesity were determined. As a result, 6 months of 500mg GSH supplementation did not increase intracellular GSH concentration. No intervention effects were observed on immune cell distribution, inflammatory cytokines secreted by PBMC after 24 h stimulation with PHA-L, neutrophils respiratory burst and clinical parameters.

GSH Supplementation and Intracellular GSH

A randomized controlled study conducted by Richie et al. showed that oral GSH supplementation increased body stores of GSH (116). The authors provided participants with 250 mg or 1000 mg Setria® GSH per day or placebo. As a result, participants taking 1000 mg per day showed an increase of total GSH in lymphocytes after 1 month of intervention. Both total GSH in plasma and lymphocytes increased after 3 month of administration. However, no changes were observed among participants who were provided with 250 mg GSH. In the current study, participants took 500 mg GSH for 4 months. At the end of study, GSH concentration in PBMC
stayed the same. The possible reason for the lack of change in intracellular GSH is that the dosage of 500 mg may not be enough, as showed by Richie that low-dose of GSH had no effect on the body stores of GSH.

Another possible reason for the absence of change is GSH concentration is the differences in characteristics of participants between these two studies. The participants were older adults with half of which were obese. As mentioned previously, both aging and obesity are related to reduced intracellular GSH concentration. Oral supplementation of GSH may not be able to correct the low GSH status in those population.

**GSH Supplementation and Immune Cell Populations**

Advanced age is associated with a decline of the functional capacity of the immune system. This likely contributes to the increased susceptibility to and severity of infectious, cancerous, and autoimmune diseases, which characterize the elderly population.

In the innate immune system, with increasing age, cells of the number of Langerhans cells in the skin and plasmacytoid dendritic cells declines, while the number of NK cells increases (148-150). The number of neutrophils does not change, but their phagocytic function is impaired (151). An interesting change in macrophages is that they secrete less cytokines including TNF-α and IL-6 in response to TLR1/2 (152).

In the adaptive immune system, the number of B lymphocytes is reduced because of the impaired ability to produce new B cells by hematopoietic stem cells (153). Also, the ability of B cells to produce antibodies is affected by the aging process (154). Similar to B cells, the total number of T cells is also reduced (155). However, increased expression of NK marker CD56+ has been seen in T cells, mainly CD8+ T cells, in older adult (156). The absolute numbers and percentages of total circulating γδ-T lymphocytes have been shown to decrease in older adults.
Also, their ability to proliferate in response to different stimuli such as isopentenyl pyrophosphate are impaired during aging. Researchers have pointed out that the decline in number and reduction in ability to proliferate of γδ-T cells in the elderly is due to proneness to apoptosis (159).

As discussed in previous chapter, obesity also has detrimental impacts on immune system. The participants in the current study were older adults with most of which are overweight. The immune system in those population may be impaired. Supplementation with 500 mg GSH for 4 months may not be adequate to result in any predicted changes in this population.

In addition, this is a secondary analysis of participants that were recruited in the GSH study. The study was not powered to see differences among the four groups. Under power might be another potential cause for the lack of treatment response.

Strength and Limitations

To my knowledge, this is the first randomized clinical study that evaluates the effect of GSH supplementation on immune cell populations in older adults with or without obesity. Here, not only the response of immune cell populations was investigated, but also neutrophil respiratory burst, inflammatory mediators secreted by PBMC. One limitation of this study is that only one dosage of GSH was used. It is hard to determine if a higher dosage would show any effects or not.

Summary

In summary, this 4-month double-blind clinical study showed that supplementation of 500 mg GSH was not able to correct the low GSH concentration in PBMC and to modulate immune cell population in older adults. No anti-inflammatory effects were observed. However,
a higher dosage of GSH may show different results. Since ROS plays an important role in the progression of inflammation (160), GSH as a major scavenger of ROS should be able to reduce inflammation. Studies using a higher dosage and/or younger adults with obesity were suggested to investigate the effects of oral GSH supplementation on obesity-induced inflammation.
Table 4-1. Demographics and baseline clinical characteristics of study participants.

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>NC</th>
<th>OC</th>
<th>NG</th>
<th>OG</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>102</td>
<td>26</td>
<td>24</td>
<td>25</td>
<td>27</td>
<td>N/A</td>
</tr>
<tr>
<td>Age (y)</td>
<td>59.5 ± 0.6</td>
<td>59.5 ± 1.3</td>
<td>60.7 ± 1.3</td>
<td>59.3 ± 1.2</td>
<td>58.5 ± 1.1</td>
<td>0.63</td>
</tr>
<tr>
<td>Gender (n, %)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.79</td>
</tr>
<tr>
<td>Male</td>
<td>41 (40.2%)</td>
<td>10 (38.5%)</td>
<td>11 (45.8%)</td>
<td>11 (44.0%)</td>
<td>9 (33.3%)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>61 (59.8%)</td>
<td>16 (61.5%)</td>
<td>14 (54.2%)</td>
<td>14 (56.0%)</td>
<td>18 (66.7%)</td>
<td></td>
</tr>
<tr>
<td>Blood Pressure</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>130.4 ± 1.6</td>
<td>123.0 ± 3.7</td>
<td>136.0 ± 2.0</td>
<td>126.6 ± 3.3</td>
<td>136.0 ± 3.1</td>
<td>0.01</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>79.3 ± 1.0</td>
<td>72.5 ± 1.9</td>
<td>86.0 ± 1.5</td>
<td>76.1 ± 2.0</td>
<td>82.9 ± 1.7</td>
<td>0.00</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>84.0 ± 2.1</td>
<td>66.9 ± 2.1</td>
<td>99.3 ± 3.3</td>
<td>70.3 ± 2.4</td>
<td>99.6 ± 3.3</td>
<td>0.00</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>29.5 ± 0.6</td>
<td>24.2 ± 0.5</td>
<td>34.0 ± 0.8</td>
<td>24.4 ± 0.7</td>
<td>35.4 ± 0.9</td>
<td>0.00</td>
</tr>
<tr>
<td>WC (cm)</td>
<td>102.3 ± 1.7</td>
<td>89.5 ± 1.6</td>
<td>113.5 ± 3.8</td>
<td>92.2 ± 1.7</td>
<td>114.2 ± 2.3</td>
<td>0.00</td>
</tr>
<tr>
<td>SAD (cm)</td>
<td>22.1 ± 0.4</td>
<td>18.7 ± 0.4</td>
<td>22.1 ± 0.4</td>
<td>22.1 ± 0.4</td>
<td>25.4 ± 0.9</td>
<td>0.00</td>
</tr>
<tr>
<td>TC (mg/dL)</td>
<td>192.5 ± 3.9</td>
<td>195.6 ± 6.5</td>
<td>189.0 ± 9.1</td>
<td>193.0 ± 7.6</td>
<td>192.0 ± 8.1</td>
<td>0.95</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>116.0 ± 7.0</td>
<td>104.2 ± 11.7</td>
<td>154.0 ± 19.7</td>
<td>87.2 ± 9.3</td>
<td>121.7 ± 11.2</td>
<td>0.01</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>56.7 ± 1.7</td>
<td>61.2 ± 2.8</td>
<td>50.3 ± 3.7</td>
<td>63.3 ± 3.9</td>
<td>51.5 ± 2.5</td>
<td>0.01</td>
</tr>
<tr>
<td>Non-HDL (mg/dL)</td>
<td>136.1 ± 3.9</td>
<td>131.4 ± 6.8</td>
<td>139.0 ± 9.1</td>
<td>132.3 ± 8.2</td>
<td>141.7 ± 7.6</td>
<td>0.74</td>
</tr>
<tr>
<td>LDL (mg/dL)</td>
<td>118.5 ± 4.2</td>
<td>114.7 ± 7.2</td>
<td>114.4 ± 8.7</td>
<td>129.9 ± 9.8</td>
<td>118.1 ± 7.8</td>
<td>0.61</td>
</tr>
<tr>
<td>GLU (mg/dL)</td>
<td>83.4 ± 1.4</td>
<td>77.7 ± 2.6</td>
<td>90.1 ± 3.3</td>
<td>79.8 ± 2.2</td>
<td>86.0 ± 2.5</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± SEM. A one-way ANOVA was performed to compared difference among the four groups at the baseline. P-values indicated that there were statistical differences among the four groups. SBP: systolic blood pressure; DBP: diastolic blood pressure; BMI: body mass index; WC: waist circumference; SAD: sagittal abdominal diameter; TC: total cholesterol; TG: triglycerides; HDL: high density lipoprotein cholesterol; non-HDL: non-high density lipoprotein cholesterol; LDL: low density lipoprotein cholesterol; GLU: glucose; CRP: C-reactive protein. NC: non-obese control group; OC: obese control group; NG: non-obese GSH group; OG: obese GSH group.
Table 4-2. Self-reported side effects, illnesses, and related behaviors of participants throughout the intervention period.

<table>
<thead>
<tr>
<th>Questions</th>
<th>NC (n=26)</th>
<th>OC (n=24)</th>
<th>NG (n=24)*</th>
<th>OG (n=27)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>During the study, did you experience any side effect(s)?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.88</td>
</tr>
<tr>
<td>Yes</td>
<td>2 (7.7%)</td>
<td>3 (12.5%)</td>
<td>3 (12.5%)</td>
<td>2 (7.4%)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>24 (92.3%)</td>
<td>21 (87.5%)</td>
<td>21 (87.5%)</td>
<td>25 (92.6%)</td>
<td></td>
</tr>
<tr>
<td>During the study, did you take two study capsules every day?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.27</td>
</tr>
<tr>
<td>Yes</td>
<td>25 (96.2%)</td>
<td>19 (79.2%)</td>
<td>22 (91.7%)</td>
<td>23 (85.2%)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>1 (3.8%)</td>
<td>5 (20.8%)</td>
<td>2 (8.3%)</td>
<td>4 (14.8%)</td>
<td></td>
</tr>
<tr>
<td>During the study, have there been any changes in your diet or exercise habits?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.90</td>
</tr>
<tr>
<td>Yes</td>
<td>6 (23.1%)</td>
<td>4 (16.7%)</td>
<td>4 (16.7%)</td>
<td>6 (26.7%)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>20 (76.9%)</td>
<td>20 (83.3%)</td>
<td>20 (83.3%)</td>
<td>21 (77.8%)</td>
<td></td>
</tr>
<tr>
<td>Which capsules do you think you have been taking during this study?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.50</td>
</tr>
<tr>
<td>GSH</td>
<td>14 (53.8%)</td>
<td>9 (37.5%)</td>
<td>14 (58.3%)</td>
<td>14 (51.9%)</td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>12 (46.2%)</td>
<td>15 (62.5%)</td>
<td>10 (41.7%)</td>
<td>13 (48.1%)</td>
<td></td>
</tr>
<tr>
<td>During the study, have you suffered from any allergies?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.64</td>
</tr>
<tr>
<td>Yes</td>
<td>10 (38.5%)</td>
<td>8 (33.3%)</td>
<td>8 (33.3%)</td>
<td>7 (25.9%)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>16 (61.5%)</td>
<td>16 (66.7%)</td>
<td>16 (66.7%)</td>
<td>20 (74.1%)</td>
<td></td>
</tr>
<tr>
<td>Have you received the flu shot this season?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.45</td>
</tr>
<tr>
<td>Yes</td>
<td>13 (50%)</td>
<td>10 (41.7%)</td>
<td>11 (45.8%)</td>
<td>17 (63.0%)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>13 (50%)</td>
<td>14 (58.3%)</td>
<td>13 (54.2%)</td>
<td>10 (37.0%)</td>
<td></td>
</tr>
</tbody>
</table>

* One participant did not complete the final questionnaire. NC: non-obese control group; OC: obese control group; NG: non-obese GSH group; OG: obese GSH group.
Figure 4-1. Overall flow of participants assessed for eligibility, enrolled in the GSH study, randomized to different treatments and analyzed statistically.
Figure 4-2. Participant compliance. A: Percentage Setria® GSH and placebo capsules consumed by participants during the 4-month study. B: GSH concentrations in $1 \times 10^7$ peripheral blood mononuclear cells (PBMC) among the four groups at baseline and the end of the study. NC: non-obese control group; OC: obese control group; NG: non-obese GSH group; OG: obese GSH group.
Figure 4-3. The percentage of immune cell populations in the total peripheral blood mononuclear cell among the 4 groups at baseline and the end of the study. NC: non-obese control group; OC: obese control group; NG: non-obese GSH group; OG: obese GSH group.
Figure 4-4. Respiratory burst assay was performed to quantify a respiratory burst response in neutrophils and monocytes by flow cytometry. NC: non-obese control group; OC: obese control group; NG: non-obese GSH group; OG: obese GSH group.
Figure 4-5. Cytokines secreted by PBMC after 24 h stimulation with PHA-L among the 4 groups at baseline and the end of the study. NC: non-obese control group; OC: obese control group; NG: non-obese GSH group; OG: obese GSH group; IL-6: interleukin 6; IL-10: interleukin 10; MIP-1α: macrophage inflammatory protein 1 alpha; TNF-a: tumor necrosis factor alpha.
Figure 4-6. Blood clinical parameters among the 4 groups at baseline and the end of the study.
NC: non-obese control group; OC: obese control group; NG: non-obese GSH group; OG: obese GSH group. TC: total cholesterol; HDL: high density lipoprotein cholesterol; LDL: low density lipoprotein cholesterol; TG: triglyceride; non-HDL: non-high density lipoprotein cholesterol; GLU: glucose.
Figure 4-7. Anthropometric measurements among the 4 groups at baseline and the end of the study. NC: non-obese control group; OC: obese control group; NG: non-obese GSH group; OG: obese GSH group.
CHAPTER 5
AGED GARLIC EXTRACT AND GSH REDUCE LPS-INDUCED INFLAMMATION IN ADIPOCYTES THROUGH INHIBITING ACTIVATION OF NF-κB AND MAPK PATHWAYS

Introduction

Obesity remains a global health concern as it is a major risk factor for many metabolic diseases, including, type 2 diabetes, cardiovascular diseases and certain cancers (161). Recently, a chronic low-grade inflammation in obesity, also called obesity-induced inflammation, has been considered the link between obesity and obesity-associated metabolic diseases (4).

The cause of this obesity-induced inflammation is not clear yet. Elevated circulating free fatty acids (FFAs) often found in obesity (162) may play a role in the development of obesity-induced inflammation. Evidence showed that FFA can activate the pro-inflammatory NF-κB pathway, resulting in increased release of pro-inflammatory cytokines, such as TNF-α, IL1-β and IL6 and MCP-1 (163). The possible mechanisms by which FFAs induce the chronic inflammation are displayed in Figure 5-1. As lipids accumulate in the body during obesity, FFA concentration increases in plasma. This in turn induces activation of NADPH oxidase and increases production of ROS through the Toll like receptor 4 (TLR4) on the surface of cells (164, 165). ROS is a group of chemically reactive molecules derived from molecular oxygen, including hydrogen peroxide (H₂O₂), superoxide anion (O₂⁻), hydroxyl radical (•OH) and singlet oxygen (¹O₂), etc. These molecules are metabolic by-products generated in cells in small amounts and cleared by antioxidants. When the production exceeds antioxidants capacity, it will impair normal cell structures, and may activate unexpected signaling pathways. In 1991, Schreck et al. had demonstrated that H₂O₂ can activate NF-κB by directly adding H₂O₂ into the cell culture media(166). Later studies showed that NF-κB was activated through different
mechanisms in different cell types (167-169). However, IκB phosphorylation is required for translocation of NF-κB from cytosol to nucleus (170).

ROS can also activate the MAPK signaling pathway in which the three major well-studied pathways are the ERK, JNK and p38 kinase pathways (171-173). Although those kinases are activated through different cascades, their phosphorylated forms activate AP1, another nuclear translational factor. AP 1 is usually in the form of heterodimer of c-Fos and c-Jun proteins. Activation of AP 1 leads to an increase in the expression of pro-inflammatory cytokines.

Given the important role that ROS plays in the development of inflammation and the results observed from the human studies, this in vitro study was conducted to investigate potential mechanisms underlying the anti-inflammatory effects of AGE and GSH in obese or overweight adults. To elucidate this mechanism, 3T3-L1 adipocytes were used. It is hypothesized that pretreatment of 3T3-L1 adipocytes with AGE or GSH would reduce LPS-induced activation of the NF-κB and MAPK signaling pathway.

**Materials and Methods**

**Aged Garlic Extract and GSH Solution Preparation**

A stock solution of AGE (100 mg/mL) was prepared by dissolving a 1000mg AGE caplet (Wakunaga of America, CA, USA) overnight in 10 mL HCl at pH = 2.0. The digested solution was then centrifuged at 14,000 rpm for 20mins. Supernatants were collected and adjusted to a pH of 7.4 using 1M NaOH. The stock solution of AGE was then sterilized using a 0.2µ syringe filter and stored at -80°C.
A stock solution of GSH (10 mM) was prepared by dissolving GSH (Sigma, St. Louis, MO) in deionized water. The solution was also sterilized using a 0.2 micron syringe filter and stored at -80°C.

**Cell Culture and Differentiation**

3T3-L1 mouse fibroblast cells were obtained from the American Type Culture Collection (ATCC® CL173™, Rockville, MD), cultured in Dulbecco’s Modified Eagle Medium (DMEM) (Cellgro, Manassas, VA) containing 4.5 g/L glucose and 584 mg/L L-glutamine (Cellgro, Manassas, VA), supplemented with 10% calf serum (Hyclone, Logan, UT) and Penicillin (100 U/mL) / Streptomycin (100 µg/mL) (Sigma, St. Louis, MO), and incubated at 37°C in 5% CO₂/95% humidified air. The medium was changed every 48 h until the cells reached confluence. Then, cells were plated into wells of a 24 (2 x 10⁴ per well) or 6-well plate (8 x 10⁴ per well). Two days after confluence, differentiation of 3T3-L1 preadipocytes was initiated by using DMEM supplemented with 10% fetal bovine serum (FBS), 1.5 µg/mL insulin, 0.5 mM 3-isobutyl-1-methylxanthine and 1 µM dexamethasone (differentiation medium I) for 48 h. The medium was then replaced with differentiation medium II (DMEM supplemented with 10% FBS, 1.5 µg/mL insulin) and changed every other day until the adipocytes were fully differentiated.

**Study Design**

Mature 3T3-L1 cells were pretreated with different doses of AGE (0, 2, 4, 6 and 8 mg/mL) or GSH (0, 0.5, 1, 2 and 4 mM) for 24 h, followed by 1h incubation with lipopolysaccharide (LPS). Cells were then kept in the fresh growth medium for 24 h. Supernatant were collected and stored at - 80 °C for determining the concentration of IL-6, TNF-α, MCP-1 and adiponectin.
Based on results from the dose response step, matured 3T3-L1 cells were pretreated with the optimum dose of AGE (4mg/mL) or GSH (1mM) for 24 h, followed by 1h incubation with LPS. Then cells were harvested for determining expression of cytokine genes by real-time PCR and expression of proteins in NF-κB and MAPK signaling pathways by western blot.

**Inflammatory Mediators Determination**

Multiplex immunoassays (Luminex) were used to determine concentrations of IL-6, TNF-α, MCP-1 and adiponectin in cell culture supernatant.

**Total GSH Concentration Determination**

Total intracellular GSH was measured colorimetrically from 3T3-L1 cell homogenates using a commercial GSH assay kit (Cayman, Ann Arbor, MI, USA) following kit directions.

**RNA Extraction and Quantitative Real-time PCR**

Cells were collected and lysed in 1mL cold RNAzol RT lysis buffer (Molecular Research Center, Inc., Cincinnati, OH). For analysis, cell lysates were centrifuged at 12,000 g for 5 min at 4°C. And residual lipids were removed. RNA was then isolated according to the manufacturer’s protocol. cDNA was synthesized using a high-capacity reverse-transcription kit (Applied Biosystems, Foster City, CA), and RT-qPCR was performed using SYBR® Select Master Mix (Applied Biosystems, Foster City, CA) following manufacturer’s instructions. The standard curve method was used for quantitation of results. mRNA concentrations were standardized to the housekeeping gene RPL13a. Primers were designed using the NCBI Primer BLAST tool and obtained from Eurofins Genomics, Louisville, KY. The sequences are shown in Table 5-1.

**Immunoblotting**

Nuclear and cytoplasmic fraction of p65 were determined to investigate potential effects of AGE and GSH on the NF-κB pathway by western blot. Nuclear and cytoplasmic fraction of
AP1 were assessed to investigate effects of AGE and GSH on the MAPK pathway by western blot.

To obtain nuclear and cytoplasmic protein from 3T3-L1 adipocytes, cells were collected from wells and centrifuged (1000 rpm, 10 min, 4°C). Cell pellets were resuspended with 500 μL hypotonic lysis buffer and incubated on ice for 15 min. 0.5 μL 1× Triton-X was added. Cell lysates were then centrifuged at 3,000 rpm for 10 min at 4°C. Supernatant was collected as cytoplasmic fraction and stored at - 80°C. Pellets were resuspended with 200 μL RIPA lysis buffer and sonicated on ice for 5 sec. Nuclear suspension was incubated on ice for 30 min with vortex at 10 min intervals and then centrifuged at 14, 000 g at for 30 min at 4°C. The supernatant was collected and - 80°C.

Total protein concentrations determined by the Bradford method. Cellular protein (40 μg) from each sample was dissolved in SDS running buffer, boiled at 70°C for 10 min, and loaded, in duplicate, onto a 12% Bis-Tris gel (Thermo Fisher Scientific) for electrophoresis. The gel was transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA) and blocked for 1 h in TBST blocking buffer (tris-buffered saline, 0.01% Tween-20, pH 7.4) with 5% BSA. Blocked membranes were washed and probed for p65, c-Fos protein (1:1000) (Cell Signaling Technologies, Danvers, MA) at 4°C overnight. Following incubation with the primary antibody, membranes were washed and probed with anti-rabbit IgG-HRP (1:5000) (Cell Signaling Technologies) for 1 h at room temperature. As loading controls, lamin B1 for nuclear protein (1:5000) (ABclonal Science, Inc., Woburn, MA) and α-tubulin for plasmatic protein (1:5000) (Sigma) were used as the primary antibody on stripped membranes, followed by anti-rabbit IgG-HRP (1:5000) and goat anti-mouse IgG-HRP (1:5000) (Cell Signaling Technologies) for 1 h at room temperature. Membrane-bound antibodies were detected using an electrochemiluminescent
detection reagent (Bio-Rad Laboratories, Inc., Hercules, CA). Relative protein expression was
determined by densitometric analysis (Alphaview, San Jose, CA).

**Statistical Analysis**

All data are presented as mean ± SD with P<0.05 considered significant. Statistical
analyses were performed using SAS JMP, v10 (SAS Institute, Cary, NC, USA). Differences in
cytokine secretion, intracellular GSH concentration, and gene and protein expression levels
among groups were determined by one-way ANOVA with a post-hoc LSD test.

**Results**

**Inflammatory Mediators Secreted by Differentiated 3T3-L1 Cells**

Figure 5-2 shows concentrations of adiponectin, IL-6, MCP-1 and TNF-α secreted by
3T3-L1 cells at 24 h after exposure to 100ng/mL LPS. LPS stimulation inhibited secretion of
adiponectin, and induced secretion of IL-6 and MCP-1 and by differentiated 3T3-L1 cells.
Pretreatment with AGE prevented the inhibition of LPS on adiponectin secretion, but not GSH
(Figure 5-2 A). As the dose of AGE increased, the concentrations of adiponectin in the medium
increased. Moderate dosages of AGE and GSH decreased IL-6 concentration compared to the
positive control (Figure 5-2 B). Pretreatment with AGE and GSH had no impact on MCP-1
secretion. (Figure 5-2 C). A very low concentration of TNF-α was secreted by differentiated
3T3-L1 cells, even after LPS stimulation.

**mRNA Expression Levels of Cytokines in Differentiated 3T3-L1 Cells**

Consistent with protein expression (cytokine and chemokine concentrations in medium),
Real-time PCR results showed that mRNA expression levels of adiponectin decreased, while IL-
6 and MCP-1 increased in response to LPS treatment. Expression levels of TNF-α mRNA also
increased (Figure 5-3).
Compared with a positive control, AGE treatment, but not GSH, increased mRNA expression of adiponectin, even higher than the negative control. Both AGE and GSH reduced mRNA expression of IL-6 in comparison with cells treated with LPS only. Pretreatment of AGE and GSH showed no effect on TNF-α.

**Intracellular GSH Concentration**

The concentration of intracellular GSH was also examined. LPS treatment induced the depletion of GSH in 3T3-L1 cells. GSH pre-treatment prevented the decline of intracellular GSH, but not AGE (Figure 5-4).

**Expression Levels of AP1 and NF-κB**

Expression of nuclear and cytoplasmic portion of AP1 and NF-κB was determined by western blotting (Figure 5-5 and Figure 5-6). Cells pretreated with AGE and GSH inhibited LPS induced translocation of NF-κB and AP1 from the cytosol to the nucleus.

**Discussion**

In this in vitro study, the effects and mechanisms of AGE and GSH on LPS-induced inflammation in 3T3-L1 cells was assessed. After pre-treated with AGE and GSH for 24 h, 3T3-L1 cell decreased expression of IL-6 at both protein and mRNA level. Pretreatment with AGE also increased adiponectin secretion. Moreover, I demonstrated that this anti-inflammatory effect of AGE and GSH was initiated by inhibiting activation of AP1 and NF-κB.

**AGE and GSH Prevented LPS-induced Inflammation in 3T3-L1 Cells**

Firstly, the anti-inflammatory effects of AGE and GSH were examined. As expected based on the results from the human study, AGE decreased IL-6 expression at both protein and mRNA level in 3T3-L1 cells stimulated with LPS. However, a decrease of TNF-α was only seen in the human study. One possible explanation is that the most TNF-α is secreted by activated
macrophages in adipose tissue (174). Consistent with this, very little TNF-α was secreted by differentiated 3T3-L1 cells in this study. The concentrations of TNF-α in the medium were less 0.1 pg/mL even after LPS stimulation. Unexpectedly, the mRNA expression levels of TNF-α increased by 10-fold relative to control. One possible reason can be post-transcriptional modifications which inhibited the translation of TNF-α mRNA. Another potential reason may be utilization of TNF-α by the cells. TNF-α plays multiple roles in adipocytes, including lipid metabolism, mediating insulin resistance regulating leptin production and regulating biosynthesis of plasminogen activator inhibitor (175), indicating that TNF-α can be used by adipocytes. AGE also increased expression of adiponectin in 3T3-L1 cells. This is consistent with results from a double-blind, randomized controlled trial conducted by Gomez-Arbelaez et al. (133). The authors reported that AGE supplementation increased plasma adiponectin levels in patients with metabolic syndrome, although this increase of adiponectin was not observed in AGE human study due to the different characteristics of the participants.

**AGE and GSH Inhibited Activation of NF-κB and AP1**

NF-κB and MAPK are the two major signaling pathways that are associated with obesity-induced inflammation (176). NF-κB was first discovered in 1986 (177) and is considered a proinflammatory signaling pathway (178). During obesity, overproduced ROS activates NF-κB and induces expression of proinflammatory cytokines. GSH, the most abundant endogenous scavenger of ROS in the body, can inhibit NF-κB by eliminating ROS (179). Liao et al. (180) reported that GSH also suppressed NF-κB activity through glutathionylation of p65 leading to inhibition of p65 nuclear translocation. The current study evaluated the effect of two dietary supplements, AGE and GSH which could replenish GSH in the body, through NF-κB signaling pathways. As expected, the ratio of NF-κB in the nucleus to NF-κB in the cytosol was smaller in
cells pretreated with AGE and GSH compared with those treated with LPS only, indicating that fewer NF-κB molecules were translocated from cytosol to nucleus. MAPK pathway is another major pathway that is associated with inflammatory responses (181), including three major groups of MAPK cascades, ERK1/2, JNK and p38. Studies showed that ROS could also activate the MAPK pathway (171-173). AP1, a family of transcriptional factors involved in MAPK pathway, is activated and translocated from the cytosol to the nucleus when MAPK pathway is activated. Like NF-κB, pretreatment of AGE and GSH also inhibited the translocation of AP1 from the cytosol to the nucleus.

**Strengths and Limitations**

This study has certain strengths. This work extends my understanding of mechanisms observed from the human clinical studies. To my knowledge, this is the first study to investigate the effects and mechanisms of AGE and GSH on the chronic inflammation in obesity. There are also some limitations. A single cell line model was used in this study. The conditions were different from those in individuals with obesity. A co-culture cell model is suggested to use in the future. Only the transcriptional factors from the NF-κB and MAPKs pathways were assessed. This study did not demonstrated how AGE and GSH influenced the upstream activities of these pathways.

**Summary**

In summary, this in vitro study demonstrated an anti-inflammatory effect of AGE and GSH. The anti-inflammatory effect was initiated through inhibition of the NF-κB and MAPKs signaling pathways. The potential mechanism by which AGE and GSH modulate obesity-induced inflammation is displayed in Figure 5-7.
Table 5-1. Sequences of primers used for real-time quantitative PCR.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer sequence (5’-3’)</th>
<th>Product size (bp)</th>
<th>Tm</th>
</tr>
</thead>
</table>
| Adiponectin | F-TCTGACGACACAAAAAGGGC  
             | R-ACGTCATCTTCGCGATGACT | 87   | 60  |
| II-6     | F-GTCCTTCCTACCCCAATTCCA  
             | R-TGGTCTTGGTCTTGAGCCAC | 79   | 60  |
| Tnf-α    | F-ATGGCCCAGACCCCTACCA   
             | R-TTGCTACGACGTGGCTGCTACA | 73   | 60  |
| Mcp-1    | F- CCACAACCACCTCAAGCAGCT | R- AGGCATCAGTCCGAGTCA | 75   | 60  |
Figure 5-1. Free fatty acids induce inflammation in obesity. During obesity, the elevated FFA in the body induces production of ROS in the cell through TLR4. The accumulation of ROS in the cell activates NF-κB and MAPKs signaling pathways, inducing expression of inflammatory genes.
Figure 5-2. Concentrations of cytokines secreted by differentiated 3T3-L1 cells in cell culture medium. Full differentiated, cells were pretreated with different dosage of AGE or GSH for 24 h and stimulated with 100ng/mL of LPS for 1 h. Cells were then kept in fresh growth medium for 24 h after LPS treatment. Data are presented as the mean ± SD.
Figure 5-3. mRNA expression levels of Adiponectin, IL-6, TNF-α and MCP-1 genes in differentiated 3T3-L1 cells. Fully differentiated cells were pretreated with 4 mg/mL AGE or 1 mM GSH for 24 h and stimulated with 100 ng/mL of LPS for 1 h. Data are presented as the mean ± SD.
Figure 5-4. Concentrations of intracellular GSH in differentiated 3T3-L1 cells. Fully differentiated cells were pretreated with 4 mg/mL AGE or 1 mM GSH for 24 h and stimulated with 100 ng/mL of LPS for 1 h. Data are presented as the mean ± SD.
Figure 5-5. Expression of NF-κB in nucleus and cytosol of 3T3L1 cells. Fully differentiated cells were pretreated with 4 mg/mL AGE or 1 mM GSH for 24 h and stimulated with 100 ng/mL of LPS for 1 h. Data are presented as the mean ± SD.
Figure 5-6. Expression of activator protein 1 in nucleus and cytosol of 3T3L1 cells. Fully differentiated cells were pretreated with 4 mg/mL AGE or 1 mM GSH for 24 h and stimulated with 100 ng/mL of LPS for 1 h. Data are presented as the mean ± SD.
Figure 5-7. Potential mechanism by which AGE and GSH reduces obesity-induced inflammation. AGE and GSH supplementation replenishes intracellular GSH concentration. The elevated GSH eliminates ROS that are accumulated in the cell during obesity through redox reaction and inhibits activation of NF-κB and MAPKs pathways.
CHAPTER 6
CONCLUSIONS AND FUTURE WORK SUMMARY

Conclusions

This research project focused on investigating the effects and mechanisms of consuming sulfur-containing dietary supplements on chronic inflammation in adults with obesity. The rationales behind this project are as follow. Patients with obesity are often in a chronic state of low-grade obesity-induced inflammation. Recently, this chronic inflammation is considered a link between obesity and its related chronic diseases (4). Thus, if the inflammation in obesity was attenuated, fewer complications might appear. The key is to understand how this obesity induced-inflammation occurs. The mechanisms are not clear yet. One potential origin is oxidative stress. In obesity, the elevated free fatty acid can induce production of ROS in cells by activating NADPH oxidase through TLRs. GSH, the most abundant endogenous scavenger of ROS, is diminished in subjects with obesity (6, 7). Any strategies to replenish GSH level in the body may help to attenuate obesity-induced inflammation. Given this understanding, I hypothesized that consuming sulfur-containing supplements would reduce obesity-induced inflammation and conducted two randomized placebo-controlled clinical trial and one cell culture based experiment. The dietary supplements used are Aged Garlic Extract (AGE) (Wakunaga of America Co.) and Setria® GSH (Kyowa Hakko Bio Co.).

In the human studies, six weeks of AGE (3.6 g/day) consumption modulated immune cell distribution, decreased serum TNF-α (p = 0.04) and IL-6 (p = 0.05) concentrations and reduced blood LDL (p = 0.05) concentration in adults with obesity, indicating that the consumption of AGE can produce anti-inflammatory effects in adults with obesity. In contrast, four months of GSH (500mg/day) consumption did not show anti-inflammatory effects. The reasons can be
insufficient dose of GSH, characteristics of participants, and underpowered statistics in the GSH study. In addition, AGE also contains many other compounds including S-methylcysteine, S-ethyl cysteine and S-propyl cysteine, and F-4 protein fraction which may counteract the inflammation through cascades other than GSH,

In the in vitro study, both AGE and GSH were used to pre-treat differentiated adipocyte (3T3-L1) prior to stimulation of inflammation with LPS. Cells pre-treated with AGE and GSH decreased the expression of IL-6 at both protein and mRNA level. In addition, AGE pre-treatment also increased the expression of adiponectin. My further studies investigated the two major inflammatory signaling pathways. AGE and GSH pre-treatment inhibited translocation of NF-κB and AP1 from the cytosol to the nucleus, both of which are two major transcriptional factors involved in the inflammatory signaling pathways.

The in vitro confirmed the anti-inflammatory effect of AGE observed in the human study. However, there are certain inconsistencies between the human studies and the in vitro study. Unlike the AGE study, AGE pretreatment also increased expression of adiponectin in 3T3-L1 cells. The possible reason is because the participants in the human study were adults with obesity but metabolically healthy. Ryo et al. demonstrated a negative association between adiponectin concentration and the clinical phenotype of metabolic syndrome (132). In a double-blind, randomized controlled trial conducted by Gomez-Arbelaez et al. (133), AGE supplementation increased plasma adiponectin levels in patients with metabolic syndrome. In addition, a decrease of TNF-α was only seen in the human study. One possible explanation is that the most TNF-α is secreted by activated macrophages in adipose tissue (174). Consistent with this, very little TNF-α was secreted by differentiated 3T3-L1 cells in this study. Different from the single cell model, many other cells are involved in adipose tissue, including macrophages which secret TNF-α. The
in vitro study also demonstrated an anti-inflammatory effect of GSH, which was not observed in the GSH study. The insufficient dosage of GSH, the characteristics of the participants and the underpowered statistics may account for the lack of responses in the human study.

In summary, these data suggested that AGE and GSH can reduce the chronic inflammation in obesity by inhibiting activation of NF-κB and MAPK signaling pathways, although the anti-inflammatory effects of GSH were not seen in the human study. The take home-message of this project is that consuming sulfur-containing dietary supplements which support GSH production in the body may be an effective mode to prevent the chronic low-grade inflammation in obesity, therefore may reduce the risks of obesity related chronic disease. Further studies should be conducted to determine optimal doses of sulfur-containing dietary supplements for reducing obesity-induced inflammation.

**Strengths and Limitations**

This project has several strengths. First, this project consists of both human clinical trials and in vitro studies, examining both the clinical and mechanistic effects of AGE and GSH. The human studies showed the anti-inflammatory effects. The in vitro study confirmed those effects and extended the findings from the human studies. To my knowledge, this is the first project that investigated the effects and mechanisms of consuming sulfur-containing dietary supplement on obesity-induced inflammation.

There are also certain limitations. In the AGE study, participants were instructed to take the dietary supplement for only six weeks. It is hard to evaluate the long-term effects of AGE on obesity-induced inflammation. In addition, only one dosage of AGE was provided for the participants, which limited the appropriateness of comparison of results from the AGE study with those from previous studies, due to the variation in AGE doses. Similarly, different doses
should be tested in the GSH study. In the in vitro study, a single cell line model was used. The conditions were different from those in individuals with obesity. Moreover, only the transcriptional factors from the NF-κB and MAPKs pathways were examined. This study did not demonstrated how AGE and GSH influenced these pathways in the up-stream.

**Future Direction**

The human study on AGE indicated an anti-inflammatory effect of AGE on obesity-induced inflammation, suggesting that AGE might be used as a supporting strategy to address the social economic burden of obesity. Future studies should be conducted to evaluate the long-term effect of AGE supplementation. Also, studies combine AGE supplementation and weight management strategies are suggested to conduct in the future.

Although, the GSH study failed to demonstrate the effect of GSH on the chronic inflammation in obesity. Given to the findings from the in vitro study and the roles that GSH played in the development of obesity induced-inflammation, a higher dose of GSH might support the anti-inflammatory effect of GSH.

Results from the in vitro study suggested that AGE and GSH prevented the progression through inhibiting activation of NF-κB and AP1. Due to lack of information about how AGE and GSH affect NF-κB and MAPKs signaling pathways, future studies can focus on the upstream of these two pathways. Also, a co-culture cell model was suggested to mimic the complicated environment in the adipose tissue during obesity.
LIST OF REFERENCES


115. Nantz MIP, Rowe CA, Muller CE, Creasy RA, Stanilka JM, Percival SS. Supplementation with aged garlic extract improves both NK and gamma delta-T cell function and reduces the severity of cold and flu symptoms: A randomized, double-blind, placebo-controlled nutrition intervention. Clinical Nutrition. 2012;31(3):337-44.


149. Jing Y, Shaheen E, Drake RR, Chen NY, Gravenstein S, Deng YP. Aging is associated with a numerical and functional decline in plasmacytoid dendritic cells, whereas myeloid dendritic cells are relatively unaltered in human peripheral blood. Human Immunology. 2009;70(10):777-84.


109


BIOGRAPHICAL SKETCH

Changjie (Henry) Xu was born in Anhui, China. He attended Tianjin Medical University in August 2005, and earned his bachelor degree there in Sports Science in 2009. After graduation, Henry continued his graduate studies in Tianjin Medical University with a major in Nutrition and Food Hygiene. During that time, he followed dietitians working in the clinical settings. Besides clinical practice, Henry was also interested in basic research. During his master’s program, he worked on nephrotic syndrome with a rat model. In July 2012, he earned his master’s degree. At the meantime, he was admitted to another master program in Biochemical and Biomedical sciences at the Chinese University of Hong Kong. After completion of his study in Hong Kong, Henry started his PhD program in Nutritional Science at University of Florida. He worked with Dr. Anne Mathews and Dr. Susan S. Percival focusing on the clinical and mechanistic effects of consuming sulfur-containing dietary supplements on obesity-induced inflammation. Henry is graduating in the fall of 2017 to earn his doctorate degree in nutritional science from the College of Agricultural and Life Sciences.