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Effects of ergothioneine on endothelial cell and macrophage characteristics, and markers of atherosclerosis risk under high lipid conditions

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Abstract

Ergothioneine (EGT) is an antioxidant and potential anti-inflammatory molecule that may have protective effects against the lipid-induced oxidative stress and inflammation that occurs in the vasculature of individuals with obesity and metabolic syndrome. This thesis addresses the hypothesis that EGT supplementation decreases endothelial cell dysfunction and macrophage inflammatory characteristics under high lipid conditions, and markers of atherosclerotic risk in diet-induced obese mice. Results showed that EGT did not influence endothelial tube formation or stability but reduced nitric oxide concentration in endothelial cells. Moreover, EGT decreased the accumulation of reactive oxygen species, and potentially nitric oxide, by macrophages. In diet-induced obese mice with characteristics of metabolic syndrome, EGT did not influence circulating lipid, lipoprotein and glucose concentrations which are markers of atherosclerotic risk. In conclusion, EGT has moderate effects on vascular cells *in vitro*, and does not influence blood glucose and lipid markers of vascular disease risk in diet-induced obese mice.

Keywords

Mushrooms, ergothioneine, antioxidant, anti-inflammatory, obesity, metabolic syndrome, vascular disease, endothelial cells, macrophages

Summary for Lay Audience

Obesity is a chronic disease that puts individuals at risk for metabolic syndrome. Those diagnosed with metabolic syndrome have high levels of fats and sugar in their bloodstream, which can deposit in cells and tissues throughout the body and cause oxidative damage. Endothelial cells, which line the inner walls of blood vessels, are vulnerable to injury from these high levels of circulating fats and sugar. In addition, immune cells such as macrophages, which also circulate in the bloodstream, can accumulate excess fats, and drive chronic inflammation within blood vessel walls. As a result of both endothelial cell damage and chronic inflammation in blood vessels, vascular disease can develop. Atherosclerosis, a vascular disease, is a condition characterized by the build up of fat in blood vessel walls leading to blockages and decreased blood flow to tissues and organs which can result in heart attacks and strokes. Ergothioneine (EGT) is an antioxidant and potential anti-inflammatory molecule that may be a potential candidate to help protect against blood vessel cell stress induced by high fat. EGT is specifically found in mushrooms and there is some evidence that EGT may modify vascular disease risk by altering fat levels in the bloodstream and protecting cells against oxidative damage. This project investigated whether EGT supplementation could modify vascular disease risk under high fat conditions. EGT was shown to have moderate effects on endothelial cell and macrophage function during exposure to high fat conditions in petri dishes. Further studies in obese mice fed a high fat diet revealed no effects on body weight or characteristics of metabolic syndrome (blood fats and sugar) as markers of atherosclerosis and vascular disease risk. It is possible that the dose and duration of EGT supplementation given to obese mice were not high enough or long enough to cause significant changes in the characteristics of metabolic syndrome which increase vascular disease risk. Future studies will determine whether EGT could be used as a vascular health supplement for individuals with obesity and metabolic syndrome.

Co-Authorship Statement

Rachel Wilson assisted with feeding and weighing of the mice throughout the study (Figure 3.12) and tissue harvesting after sacrifice.

Cindy Sawyez measured plasma and liver lipids in Table 3.1 and Figure 3.13 as well as THP-1 macrophage pro-inflammatory cytokines in Figure 3.10.

Rishikesan Chandrakumar assisted with HMVEC tube formation and stability experiments under combined high fatty acid and hypoxic conditions in Figure 3.4.

Yun Jin (Carrie) Chen developed the customized, automated ImageJ protocols used for image analysis presented in Figure 3.9.

Brian Sutherland and Julia St John assisted with mouse sacrifice and tissue harvesting.

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Abbreviations and Symbols

ACAT1	Acyl coenzyme A: cholesterol acyltransferase-1
AMA	American Medical Association
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BH_4	Tetrahydrobiopterin
BSA	Bovine serum albumin
CCL20	Chemokine ligand 20
CD14	Cluster of differentiation 14
CDC	Centers for Disease Control and Prevention
CETP	Cholesteryl ester transport protein
CMA	Canadian Medical Association
COX2	Cyclooxygenase-2
Ct	Cycle threshold
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGT	Ergothioneine
ELISA	Enzyme linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase

FAD	Flavin adenine dinucleotide
FBS	Fetal bovine serum
FPLC	Fast protein liquid chromatography
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HDL	High-density lipoprotein
HMVEC	Human microvascular endothelial cells
ICAM-1	Intercellular adhesion molecule 1
IL	Interleukin
iNOS	Inducible nitric oxide synthase
LDL	Low density lipoprotein
$LDLr^{-/-}$	LDL receptor knockout
LOX-1	Lectin-like oxidized LDL receptor 1
MCP-1	Monocyte chemotactic protein 1
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
MYB	Myeloblastosis
NADPH	Nicotinamide adenine dinucleotide phosphate
NAFLD	Non-alcoholic fatty liver disease
NF-κB	Nuclear factor kappa B
NO	Nitric oxide
OCTN1	Organic cation transporter novel type-1

PA/OA	Palmitate plus oleate
PBS	Phosphate-buffered saline
PDB	Phorbol 12,13-dibutyrate
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
RPMI	Roswell Park Memorial Institute
RT-qPCR	Real-time quantitative polymerase chain reaction
SR-BI	Scavenger receptor class B type I
TBARS	Thiobarbituric acid reactive substances
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha
TRL	Triglyceride rich lipoproteins
VCAM-1	Vascular cell adhesion molecule 1
VLDL	Very low density lipoprotein
WHO	World Health Organization

Chapter 1 *

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1 Introduction

1.1 Obesity, Metabolic Syndrome, and the link to Vascular Disease

Obesity has quickly become one of the most serious public health concerns of the 21st century, with the worldwide prevalence of obesity increasing nearly 3-fold since 1975 [1]. According to the World Health Organization (WHO) in 2016, over 650 million adults in addition to over 340 million children and adolescents suffer from obesity [1]. In 2018, Statistics Canada stated that 9.9 million Canadian adults (36.3%) were classified as overweight, and 7.3 million adults (26.8%) were considered obese. Therefore, the total population of Canadian adults that suffer from excessive weight is 63.1%; an increase from 2015, when it was at 61.9% [2]. Obesity is defined as abnormal or excessive fat accumulation that presents a health risk and is diagnosed based on a body mass index (BMI) greater than or equal to 30 kg/m^2 [1]. Environmental, lifestyle, and genetic factors all contribute to the development of obesity [3]. Many organizations including Obesity Canada, the Canadian Medical Association (CMA), the American Medical Association (AMA), the Centers for Disease Control and Prevention (CDC) and the WHO now consider obesity a chronic disease [1,4–7]. Obesity is also recognized as a major risk factor for many other chronic diseases including diabetes, musculoskeletal disorders, cardiovascular disease and cancer [1]. This is a major economic issue as well with the Public Health Agency of Canada calculating the annual direct health care costs of Canadians that suffer from obesity, in constant 2001 Canadian dollars, to be $7.0 billion in 2011 and projected to rise to $8.8 billion in 2021 [8].

The excess deposition of visceral adipose tissue characterizes abdominal obesity and can be associated with downstream complications including hyperglycemia, hyperinsulinemia, and dyslipidemia [9]. In addition, abdominal obesity can increase blood pressure through renin-angiotensin-aldosterone activation in adipose tissue and sympathetic activation [10]. As a result, individuals who suffer from abdominal obesity are at increased risk of developing metabolic syndrome [11]. Metabolic syndrome is diagnosed in individuals who show 3 of the 5 following conditions: large waist circumference, ≥ 102 cm in men, 88 cm in women; high blood pressure, ≥ 130/85 mm Hg; high blood glucose levels, ≥ 5.6 mmol/L; high blood triglyceride levels, ≥ 1.7 mmol/L; and low levels of high-density lipoprotein (HDL) -cholesterol, < 1.0 mmol/L in men, < 1.3 mmol/L in women. In 2014, nearly 1 in 5 Canadian adults were considered to have metabolic syndrome [12].

A common characteristic shared by both obesity and metabolic syndrome are the elevated levels of circulating lipids and their deposition into various tissues including skeletal muscle, the liver, and vasculature [13]. Cells that are exposed to excess lipids are prone to cellular dysfunction and death [14]. Within skeletal muscle and liver tissue, lipid accumulation can lead to insulin resistance, which is a hallmark of type 2 diabetes mellitus [15]. Moreover, lipid accumulation in the liver can promote the onset of non-alcoholic fatty liver disease (NAFLD) in individuals; a disease which has been associated with a cluster of metabolic abnormalities related to metabolic syndrome [13]. Vascular disease is also prevalent in individuals who suffer from obesity and metabolic syndrome [13], and can lead to death from heart attack and stroke which are commonly attributed to the blockage of blood flow from lipid deposition within the vasculature, a condition known as atherosclerosis [16].

1.2 Endothelial Cells and Macrophages in Vascular Disease

1.2.1 Vascular disease

When individuals gain excessive weight, plasma lipid levels can increase, as is observed in metabolic syndrome [17]. Under these conditions, vascular cells, including endothelial cells and macrophages, are some of the first cell types to be exposed to high levels of circulating lipids [18,19]. The responses of these cell types to high lipid exposure can

modify the development and progression of vascular disease. In particular, the ability of endothelial cells to migrate and repair blood vessels, and to produce nitric oxide (NO) [20]; and the ability of monocytes to differentiate into macrophages, and to subsequently secrete cytokines, and produce NO and reactive oxygen species (ROS) can all impact vascular disease risk [21,22]. The processes whereby endothelial cells and macrophages contribute to the development of vascular disease under high lipid conditions are described in detail below.

Under hyperlipidemic conditions, such as obesity and metabolic syndrome, endothelial cell damage causes the endothelial wall to become leaky, allowing lipids to pass through and deposit into the intima of the artery wall via paracellular and transcytosis pathways [23]. This is considered one of the earliest events in the development of atherosclerosis [22]. Circulating low density lipoprotein (LDL) concentrations are a key risk factor for the development of atherosclerosis [23]. The functional role of LDL is to transport lipids from the liver to peripheral tissues. LDL is clinically considered the ‘bad cholesterol’ as it contains high levels of cholesterol and triglycerides and low levels of proteins [24]. In contrast, HDL is clinically considered the ‘good cholesterol’ because it transports cholesterol from peripheral tissues back to the liver for clearance. When LDL particles enter the intima, they are oxidized by reactive intermediates of molecular oxygen known as ROS [22,25]. Oxidized LDL, in addition to high blood pressure and serum triglycerides, activate endothelial cells to secrete pro-inflammatory cytokines and chemokines into the bloodstream and express adhesion molecules on their cell membrane [24,26].

Pro-inflammatory cytokines and chemokines such as monocyte chemotactic protein 1 (MCP-1) attract circulating monocytes to the vascular endothelium [24,27]. Rolling monocytes then bind to endothelial cells and migrate into the intima using adhesion molecules such as vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1) and E-selectin [24]. After migrating into the intima, monocytes differentiate into pro-inflammatory macrophages which are responsible for clearing oxidized LDL through receptor-mediated endocytosis and phagocytosis [22]. Macrophages release a variety of pro-inflammatory cytokines such as interleukin (IL)-1β, tumor necrosis factor alpha (TNF-α) and MCP-1 into the extracellular matrix (ECM), which recruits more

macrophages and further propagates inflammation as well as NO and ROS [28]. The responses to short term exposure to high lipids in macrophages include storage of lipids in lipid droplets, β-oxidation and cholesterol efflux, which can result in proper clearance [28]. However, during prolonged exposure, macrophages start to accumulate too many lipids, and as lipid uptake exceeds β-oxidation and efflux, macrophages become increasingly lipid-laden and are known as foam cells [27]. Foam cells secrete growth factors, interferon γ, matrix metalloproteinases, ROS, and pro-inflammatory cytokines to continue attracting monocytes to the vasculature and stimulating macrophage proliferation, which further amplifies the inflammatory response, leading to the progression of atherosclerosis [24]. Figure 1.1 summarizes the roll of endothelial cells and macrophages in the development of vascular disease under high lipid conditions.

1.2.2 Endothelial Cells, NO and ROS production in Vascular Disease

Endothelial cells that make up the endothelium play a crucial role in vascular homeostasis by maintaining neovascularization, vascular tone and permeability, blood fluidity, gas, nutrient and waste exchange as well as the inflammation/immune response [25,29]. In response to vascular injury or tissue ischemia, endothelial cells migrate and proliferate to initiate the formation of new blood vessels from pre-existing blood vessels [30]. Moreover, endothelial cells regulate vascular tone by a variety of vasodilators such as NO, prostacyclin and bradykinin as well as vasoconstrictors such as endothelin-1 and angiotensin-II [31].

NO provides many protective functions in endothelial cell physiology [24]. For example, NO can inhibit the expression of pro-inflammatory cytokines and chemokines, LDL oxidation and cytokine-induced expression of VCAM-1 and MCP-1 [25]. As previously mentioned, these are all important factors in the development of atherosclerosis. Endothelial nitric oxide synthase (eNOS) is responsible for the synthesis of NO via an enzymatic oxidation of arginine to citrulline which requires several cofactors including nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide, Ca^{2+}/ calmodulin, and tetrahydrobiopterin (BH_4) [32].

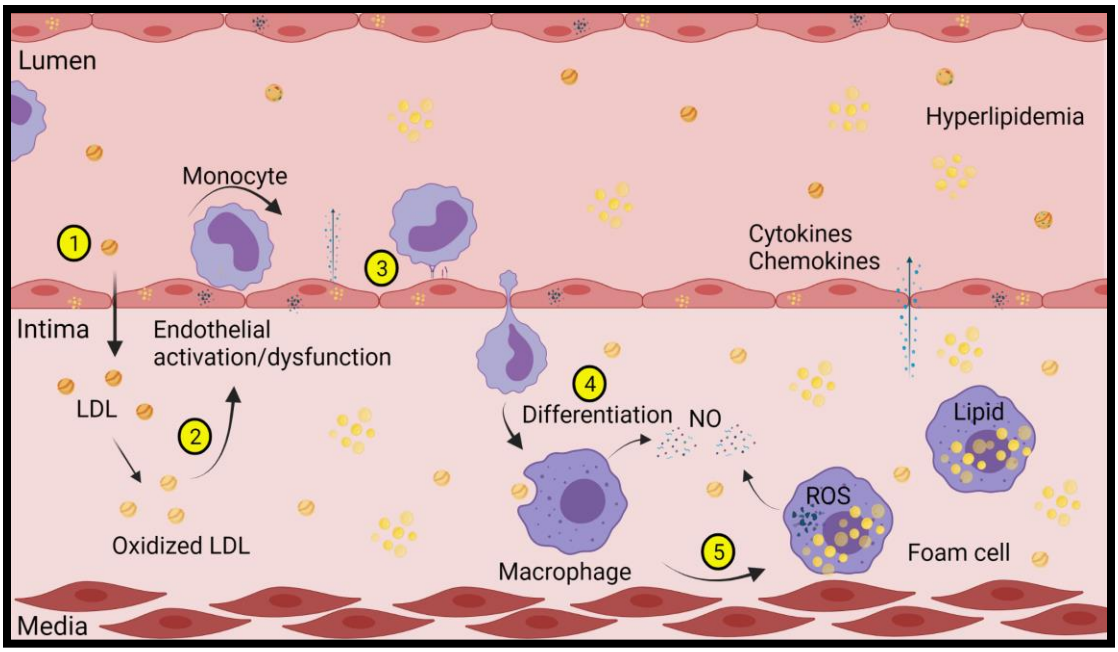


Figure 1.1 Summary of endothelial cell and macrophage responses under high lipid conditions in vascular disease

(1) During obesity and metabolic syndrome, elevated levels of circulating lipids induce endothelial cell damage which allows low density lipoprotein (LDL) particles to deposit into the intima. (2) Once in the intima, LDL particles undergo oxidation, leading to the activation of endothelial cells. These activated endothelial cells start expressing adhesion molecules at their cell surface and release pro-inflammatory cytokines and chemokines into the blood circulation. (3) In response, circulating monocytes are recruited to the vascular wall where they adhere to endothelial cells and migrate into the intima. (4) Infiltrating monocytes differentiate into macrophages, phagocytose oxidized LDL and secrete a variety of pro-inflammatory cytokines, reactive oxygen species (ROS) and nitric oxide (NO) into the extracellular matrix. This results in further recruitment of macrophages and propagation of inflammation. (5) During chronic exposure to lipids and lipoproteins such as LDL, macrophages become increasingly lipid-laden and are known as foam cells. Foam cell and lipid accumulation in the intima intensifies the inflammatory environment leading to the development and progression of atherosclerosis. Figure was created in BioRender.

After its synthesis, NO can diffuse past the cell membrane and act as a potent paracrine mediator [33]. However, NO has a very short half-life as it is rapidly metabolized into nitrite and then into nitrate [34]. NO can be released in response to ischemia or shear stress and further elevated by acetylcholine, bradykinin and serotonin [21,25]. Reduced NO bioavailability is an indicator of endothelial dysfunction, which in turn is a hallmark for obesity, metabolic syndrome, and vascular disease [17,33,35].

Endothelial cells are not metabolically programmed to process large levels of lipids compared to other cells such as adipocytes [13,36]. Saturated fatty acids such as palmitate can induce endothelial dysfunction by impairing insulin signaling, promoting inflammation and mitochondrial dysfunction and accumulating toxic lipid metabolites such as ceramide [35]. These processes in turn reduce NO bioavailability and promote the formation of ROS [35]. In the setting of atherosclerosis, oxidized LDL primarily binds to receptors located on endothelial cells known as lectin-like oxidized LDL receptor 1 (LOX-1) [37]. Oxidized LDL disrupts the production of NO as well as produces ROS by LOX-1 mediated increases in NADPH oxidase, nuclear factor kappa B (NF-κB), and mitochondrial enzymes involved in oxidative signaling [24]. Moreover, oxidized LDL promotes endothelial cell apoptosis leading to increased permeability of monocytes and lipids as well as platelet activation due to exposed collagen [24].

Levels of ROS can also be induced in endothelial cells by eNOS uncoupling. This occurs when eNOS has insufficient substrates and/or cofactors present, leading to the production of ROS instead of NO [32]. NO is vulnerable to ROS such as superoxide anions as they can either inactivate NO or react with it to form peroxynitrite [31,33]. When this occurs, not only the levels of NO decrease, but peroxynitrite can promote protein nitration leading to endothelial dysfunction and death [25]. ROS can act as important second messengers that transduce intracellular signals involved in various biological processes such as angiogenesis and host cell defenses [38,39]. However, when the level of ROS exceeds the buffering capacity of the cells antioxidant defense systems, it can lead to oxidative stress and endothelial dysfunction [40]. There are many antioxidant enzymes within endothelial cells including superoxide dismutase, catalase, glutathione peroxidase, peroxiredoxin and thioredoxin that help maintain the proper levels of ROS [25]. However, there are also a

variety of sources from which ROS can be produced including the mitochondria, NADPH oxidase, uncoupled eNOS and xanthine oxidase [40]. Superoxide anion is typically the first ROS to be generated by the partial reduction of molecular oxygen, which then gives rise to hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), and peroxynitrite ($ONOO^-$) [25]. Taken together, reduced NO bioavailability in addition to elevated ROS play a key role in endothelial dysfunction, which is a major underlying problem in the development of vascular disease.

1.2.3 Macrophages, NO and ROS production in Vascular Disease

Macrophages are a component of the innate immune system and play a critical role in controlling infections, removal of debris and dead cells, promoting tissue repair and wound healing [41]. However, they can also contribute to tissue damage during inflammatory diseases such as atherosclerosis [41]. Two major sub-populations of macrophages include inflammatory (M1) macrophages and anti-inflammatory (M2) macrophages. M1 macrophages secrete pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, and IL-12 to propagate inflammation. They also secrete high levels of NO via inducible nitric oxide synthase (iNOS) and ROS to kill pathogens [41,42]. Compared to the protective effects of NO produced by eNOS in endothelial cells, NO is produced in high amounts by iNOS in macrophages over longer periods of time to fight against foreign invaders [21]. In contrast, M2 macrophages secrete anti-inflammatory cytokines such as IL-10 and transforming growth factor (TGF)-β, which contributes to the resolution phase of inflammation, wound healing, phagocytosis of apoptotic cells, tissue remodeling and angiogenesis [42].

In the setting of obesity, macrophages are recruited to tissues with excess lipid deposition including adipose tissue, skeletal muscle, liver, and vasculature to promote inflammation with the intention of resolution. However, the chronic exposure to lipids in obesity prevents the resolution phase and leads to long lasting inflammation [43,44]. Within the vasculature, macrophages take up oxidized LDL and subsequently break down cholesteryl esters into free cholesterol and fatty acids by hydrolases and lipases in late endosomes [32]. Free cholesterol can then be secreted outside of the cell by adenosine triphosphate (ATP)-

binding cassette transporters and scavenger receptor class B type I (SR-BI) or re-esterified by acyl coenzyme A: cholesterol acyltransferase-1 (ACAT1) [45]. Cholesteryl esters are stored in lipid droplets forming foam cells to prevent free cholesterol from having cytotoxic and inflammatory effects [32]. During early vascular disease, both M1 and M2 phenotypes are present to help resolve excess lipid deposition in the form of foam cells. However, as vascular disease develops, the number of foam cells increase due to the chronic exposure to lipids and this shifts the balance towards more M1 and less M2 macrophages [22,46]. This in turn escalates the inflammatory response and increases the amount of pro-inflammatory cytokines, chemokines, ROS, and NO being produced [21].

As previously mentioned, ROS can react with NO to form reactive nitrogen species (RNS) such as peroxynitrite [25]. The main source of ROS in macrophages are NADPH oxidases, complexes which can play a significant role in the regulation of monocyte differentiation and macrophage functions [40]. Other sources of ROS in macrophages include the mitochondria, xanthine oxidase, lipoxygenase and the uncoupling of NO synthases [22]. The excessive production of ROS and peroxynitrite contribute to the vicious inflammatory cycle that promotes atherosclerotic development by oxidizing LDL, activating and promoting endothelial dysfunction, and monocyte derived macrophage recruitment, activation and apoptosis [22].

Taken together, endothelial cells and macrophages play an important role in vascular homeostasis, however, during chronic exposure to high lipid conditions they are prone to oxidative stress and inflammation. Individuals who suffer from diseases such as obesity and metabolic syndrome are associated with conditions of endothelial dysfunction, atherogenic dyslipidemia, insulin resistance, and chronic low-grade inflammation [17]. As a result, it is important that steps be taken to find ways to combat the detrimental effects of excess lipid on vascular endothelial cells and macrophages in order to reduce vascular disease risk.

1.3 Ergothioneine

1.3.1 Mushrooms and Ergothioneine

The medicinal use of mushrooms have been documented since the Neolithic and Paleolithic eras, and in the modern world, medicinal mushrooms have a long-standing history of use in Asian countries [47]. Within the past decade, interest in plant-based diets in Westernized countries has brought more widespread attention to the role of mushrooms in the prevention and treatment of chronic diseases [48]. There are approximately 12000 species of mushrooms worldwide, of which about 2000 are edible. Current estimates suggest that nearly 200 wild species are used therapeutically [49]. Mushrooms contain an abundance of potential nutraceutical compounds including polysaccharides (β-glucans), vitamins, terpenes, ergosterol and polyphenols [50]. However, much recent interest has focused on L-Ergothioneine (EGT), which is particularly enriched in and considered relatively unique to mushrooms [51].

Over the last decade, the naturally occurring modified amino acid, EGT, has gained much attention as a potential therapeutic compound [51–54]. EGT is a thiol derivative of histidine and was first discovered by Charles Tanret in 1909 in the ergot fungus, *Claviceps purpurea* (Figure 1.2) [55]. Although EGT is widely distributed within tissues of both plants and animals, it is exclusively synthesized in non-yeast fungi, actinomycete bacteria, lactobacillus bacteria, and some cyanobacteria [56–59]. Plants are able to absorb EGT through their roots via a symbiotic relationship with fungi present in soil [60]. The concept that animals absorb EGT solely from the diet was initially recognized through a study of grain-fed versus casein-fed pigs, which showed that only grain-fed pigs had detectable concentrations of EGT in the blood [61]. In the early 2000s, it was established that mushrooms are the most abundant dietary source of EGT, with concentrations ranging between 0.21 and 2.6 mg/g of dry weight depending on mushroom species [62] while other dietary sources include some meat products, oat bran and beans [60]. Mushrooms of the genus *Pleurotus*, commonly known as oyster mushrooms, appear to contain the highest amount of EGT, whereas white button mushrooms *Agaricus bisporus* contain lower concentrations but are also consumed in larger quantities. A typical serving of whole mushrooms can provide between 1 and 26 mg of EGT [62]. Recent estimates of EGT intake

in general populations of European countries and the United States suggest averages between 0.051 - 0.255 mg EGT/kg body weight/day, with Italy having the highest consumption [63]. Several groups have demonstrated the bioavailability of EGT from mushroom sources [64–66]. Consumption of 100 g of white button mushrooms for 4 months resulted in significantly increased EGT concentration in the blood [67]. Furthermore, consumption of a single dose of 16 g of mushroom powder has been reported to increase EGT concentration in red blood cells [64]. Therefore, mushrooms and mushroom powders represent a unique food source of bioavailable EGT.

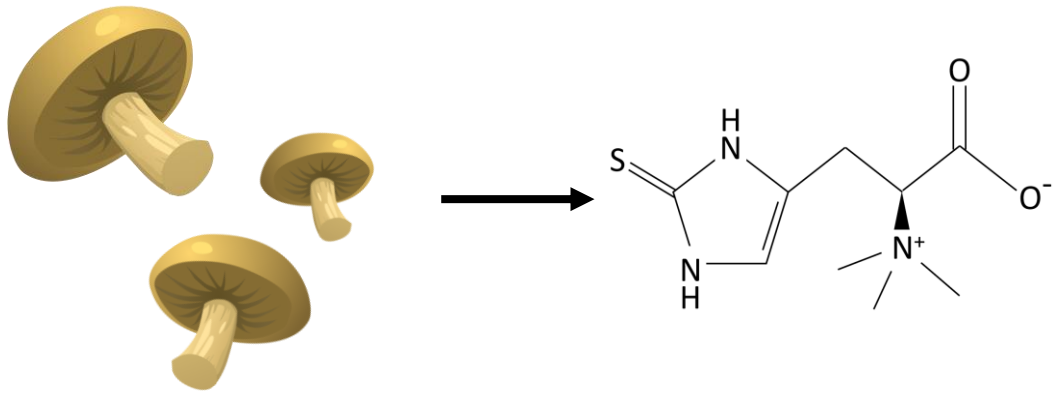


Figure 1.2 Molecular structure of L-Ergothioneine

EGT is a unique thiol derivative of histidine that was first discovered by Charles Tanret in 1909 in the ergot fungus *Claviceps purpurea* [55]. The synthesis of EGT only occurs within non-yeast fungi, actinomycete bacteria, lactobacillus bacteria, and some cyanobacteria. As a result, animals and humans rely on dietary mushrooms as their primary source of EGT.

1.3.2 Tissue Accumulation and Metabolism of Ergothioneine

The discovery that EGT can accumulate in tissues upon mushroom consumption sparked interest in the development of a sustainable supply of synthetic EGT and similar biomimetics. The synthesis of enantiomerically pure L-(+)-EGT was first described by Xu and Yadan in 1995 [68]. Since then, nature-identical biomimetics of EGT have been produced for commercial use (Mironova Labs Inc., Tetrahedron, Blue California). Recently, synthetic EGT marketed as Ergoneine® (Tetrahedron) has been approved as a safe, novel supplement with a recommended daily dose of 30 mg/day for adults and 20 mg/day for children by the European Food Safety Authority [69], and ErgoActive (Blue California) was recognized as safe by the Food and Drug Administration in the United States [70]. Although the absence of toxicity at high millimolar concentrations has been shown in rodent models [71], studies in human subjects evaluating the safety and efficacy of EGT supplementation are limited.

The plasma membrane is impermeable to EGT and uptake is dependent on the presence of organic cation transporter novel type-1 (OCTN1) encoded by the *SLC22A4* gene [72]. The significance of OCTN1-mediated transport of EGT into various tissues in health and disease has been recently reviewed [51,54]**.** OCTN1 is a highly selective Na^+- and pH-dependent transporter of EGT, which has low selectivity for other compounds, including the structurally similar intermediate product, hercynine [73]. The expression of OCTN1 is found in a variety of tissues including the liver, small intestine, kidney and smooth muscle as well as cell types such vascular endothelial cells and macrophages [74–77]. Silencing of *SLC224A* has been shown to inhibit EGT uptake and whole body knock-out of OCTN1 in mice was associated with complete absence of EGT in both blood and tissue, confirming an absence of alternative EGT uptake mechanisms [78].

In animals, EGT has a wide tissue distribution, with tissue concentrations ranging from 100 µM – 2 mM [53]. EGT is most abundant in the liver, erythrocytes, kidney, intestines, eye, bone marrow, seminal fluid, and lens of the eyes [79]. Interestingly, EGT tends to accumulate in tissues exposed to oxidative stress [52] and tissues at risk in some human

diseases including fatty and fibrotic livers and infarcted hearts [80,81], leading Halliwell et al. (2018) to suggest that EGT is an “adaptive” antioxidant [51].

Many studies have described the accumulation of EGT from the diet [53,64,67], however, there are fewer studies characterizing EGT accumulation following administration of the pure compound. Cheah et al. (2017) were the first to report that following administration of pure EGT to humans, plasma EGT levels significantly increased during the administration period in a dose-dependent manner [66]. In mice, basal EGT concentrations are highest in liver, followed by whole blood, spleen, kidney, lung, heart, small intestine, eye, large intestine then brain. After 7 days of EGT supplementation, most mouse tissues exhibit increased EGT content. Interestingly, however, after one day of EGT supplementation, only the liver and large intestine show increased EGT content, suggesting that accumulation occurs more rapidly in liver than in whole blood [79]. Similar results were found using a mouse model of chronic kidney disease, where the liver retained EGT levels despite impaired intestinal absorption [82]. EGT has also been shown to accumulate in guinea pig liver [80].

Little is known about EGT metabolism *in vivo*, however, some studies have suggested three main by-products of EGT metabolism – hercynine, EGT-sulfonate and S-methyl EGT [79]. Hercynine appears to be the predominant metabolite of EGT, and it may be the most important metabolite *in vivo* [66,79]. Unlike many diet-derived polyphenols and anti-oxidants, which are rapidly metabolized and excreted, EGT is highly retained, with relatively slow metabolic clearance [51,83]. Most recently, the high retention and slow excretion rate of EGT was demonstrated in humans following an oral dose of pure EGT [66]. These pharmacokinetic characteristics have been attributed to renal reabsorption of EGT [78,79].

1.4 Ergothioneine, Metabolic Syndrome and Vascular Disease

Numerous *in vitro* studies have demonstrated the ability of EGT to act as a general cytoprotectant with its various abilities to scavenge ROS and RNS, protect against

ultraviolet and gamma radiation induced damage, chelate metal cations (Cu^{2+}, Fe^{2+}), and elicit anti-inflammatory effects. EGT is well known for its potential antioxidant capabilities as it scavenges hydroxyl radicals, singlet oxygen, hypochlorous acid, lipid peroxides, peroxynitrite and superoxide ions (one of the most harmful derivatives of molecular oxygen for the vascular endothelium). These activities have been extensively reviewed elsewhere [51,54,84]. Interestingly, EGT is more effective at scavenging peroxynitrite and singlet oxygen than glutathione [85,86]. Moreover, EGT accumulates intracellularly within mitochondria and nuclei to confer protection against oxidative DNA damage [52]. Animal studies have suggested that EGT may only play a role in tissues and cells that express high levels of oxidative stress, leaving basal ROS levels unchanged in healthy tissues [51]. The concept of EGT as an anti-inflammatory molecule arose from case-control studies which suggested that individuals susceptible to chronic inflammatory diseases, including Crohn's disease, ulcerative colitis and type 1 diabetes, have polymorphisms in the *SLC22A4* gene encoding OCTN1 [87–89]. Studies in cell cultures have shown that EGT can decrease pro-inflammatory cytokines IL-6, IL-1β and TNF-α [90–92]. However, fewer studies exist that elucidate the potential anti-inflammatory effects of EGT *in vivo*. Some clinical studies indicate that subjects with various diseases have decreased levels of EGT in certain tissues relative to control subjects [78,82]. Taken together, this evidence suggests that raising tissue levels of EGT may be beneficial in conditions involving chronic oxidative stress and inflammation, including obesity, metabolic syndrome and, as described in the previous sections, vascular disease.

White button mushroom comprises 35 - 45% of total worldwide edible mushroom consumption and is widely cultivated throughout Europe and North America [93]. In a retrospective study of adults with pre-diabetes and two or more confirmed metabolic syndrome criteria, daily consumption of a standard serving of white button mushrooms (100 g, ~3.2 mg of EGT) for 16 weeks was associated with decreased systemic oxidative stress and inflammation. At the end of the 16-week dietary intervention, serum EGT concentrations were increased 2-fold compared to baseline, which was associated with a decrease in markers of oxidative stress and inflammation. Specifically, serum advanced glycation end products (carboxymethyl lysine, methylglyoxal derivatives) were reduced,

while increases were observed in oxygen radical absorbance capacity, an indicator of antioxidant response, and in adiponectin, an anti-inflammatory hormone [67].

As mentioned earlier, vascular disease is a common complication in individuals with metabolic syndrome, one characteristic of which is elevated blood glucose. Several *in vitro* studies indicate that EGT may protect the endothelium against glucose-induced oxidative stress. In endothelial cells treated with high glucose, pyrogallol, xanthine oxidase plus xanthine, hydrogen peroxide, and paraquat dichloride (all known inducers of oxidative stress), EGT decreased ROS production, improved cellular redox status, and increased cell viability [74,84]. EGT has also been shown to attenuate the reduction of acetylcholine-induced vasodilation in isolated rat arteries exposed to a variety of inducers of oxidative stress, and improve vasoresponsiveness of isolated basilar arteries from streptozotocin-induced diabetic rats [74]. Furthermore, EGT accumulates in endothelial cells through OCTN1 but is quickly depleted after treatment with OCTN1 siRNA. In endothelial cells, the protective effects of EGT against pyrogallol were abolished after OCTN1 siRNA treatment, clearly indicating that cellular uptake and accumulation of EGT is required. The antioxidant and cytoprotective effects of EGT could be attributed to its ability to directly scavenge ROS, downregulation of the ROS producing enzyme, NADPH oxidase 1, or the induction of antioxidant enzymes, including glutathione reductase, catalase and superoxide dismutase [74]. D'Onofrio et al. (2016) reported similar findings, that EGT protected against high glucose-induced ROS production, cell senescence, and reduced cell viability. Furthermore, observations of endothelial cell cytotoxicity in conjunction with reduced EGT content in endothelial cells during high glucose exposure support the possibility of an important role for EGT in protecting against endothelial cell cytotoxicity during hyperglycemia. The mechanism whereby EGT protects against glucose-induced endothelial cell senescence may involve upregulation of sirtuins 1 and 6, which act to downregulate the adaptor protein p66Shc and the pro-inflammatory transcription factor NF-κB, respectively [94]. Sirtuins are a family of histone deacetylases responsible for the control of cellular metabolism and stress responses linked to cellular lifespan [95,96]. Sirtuin 1 can regulate eNOS, leading to decreased hyperglycemia-induced endothelial dysfunction [94,97]. Moreover, sirtuin 6 deficiency leads to increased expression of the pro-inflammatory cytokine IL-1β and IL-6 [98]. Overall, these studies in cultured

endothelial cells and isolated small vessels suggest that EGT may be an effective modulator of cellular oxidative stress, inflammatory, and survival pathways in vascular cells.

Cardiovascular disease refers to disorders of the heart and blood vessels including coronary artery disease, heart failure, peripheral artery disease, and stroke [99]. In a recent population-based prospective study, Smith et al. (2020) proposed that identifying plasma metabolites associated with health-conscious food patterns could reveal specific metabolites that help to predict cardiovascular disease and mortality. Using liquid chromatography-mass spectrometry, 112 plasma metabolites were identified in more than 3000 participants. During a median follow-up time of 21 years, EGT had the strongest association to health-conscious food patterns and was an independent predictor for lower risk of coronary artery disease, strokes, cardiovascular mortality, and overall mortality [100]. The mechanisms underlying the potential benefit of EGT consumption in improving cardiovascular disease risk may be related to modulation of atherosclerosis development and progression described below.

Several studies have suggested that EGT-containing mixtures and EGT may protect against the initial stages of atherosclerosis by mitigating LDL oxidation, monocyte adhesion to endothelial cells, and processes that contribute to foam cell formation. Crude aqueous grey oyster mushroom extract, containing EGT, can protect against hydrogen peroxide-induced human aortic endothelial cell death. Moreover, this mushroom extract reduced the formation of conjugated dienes and thiobarbituric acid reactive substances (TBARS) in endothelial cells, which are involved in the initial and late stages of human LDL oxidation, respectively [101]. Interestingly, studies determining the bioavailability of EGT in humans from brown cremini mushroom powder showed a trend towards reducing the postprandial triglyceride response [64]. Postprandial triglycerides can increase the risk of coronary artery disease by contributing to the formation of foam cells. Furthermore, IL-1β-induced expression of the cell surface adhesion molecules V-CAM1, I-CAM1 and E-selectin was suppressed by EGT in human aortic EC. Consequently, IL-1β-stimulated binding of human monocytes to endothelial cells was reduced in co-culture experiments [102].

Beyond its potential effects on atherosclerosis, EGT may modulate the development and progression of vascular disease through direct effects on vascular function. Studies by Gokce et al. (2014) have suggested a role for EGT in promoting aortic relaxation [103]. This appeared to be endothelium-dependent, as the effect was abolished in denuded aortic ring preparations or by pre-treatment with a NO synthase inhibitor. Pre-treatment with EGT did not affect acetylcholine-induced relaxation in either intact or endothelium-denuded preparations, suggesting that EGT does not interfere with basal or agonist-stimulated production of NO. Follow-up studies indicated that EGT prevents the reduction of acetylcholine-induced relaxation of aortic rings caused by diethyldithiocarbamate and hypoxanthine plus xanthine oxidase, both potent inducers of superoxide formation [103]. Taken together, these studies suggest that EGT may be an independent predictor for lower risk of cardiovascular disease which could be attributed to the protective effects of EGT in initial stages of vascular disease described in section 1.2.

Overall, EGT is a unique compound that accumulates in high concentrations within a variety of tissues, especially those that express oxidative stress [52]. The uptake of EGT by humans primarily depends on the consumption of mushrooms as they contain the highest amount of EGT within the diet [54]. Studies have shown that EGT has antioxidant and potential anti-inflammatory properties within cells under cellular stress [52,90,103–105]. Conditions involving chronic oxidative stress and inflammation, including obesity and metabolic syndrome [17], may be potential targets for EGT. Interestingly, studies that have been recently described above suggest that EGT may play a protective roll against vascular disease. Taken together, EGT may be a potential candidate to protect against vascular disease observed in individuals that suffer from obesity and metabolic syndrome. Figure 1.3 identifies the potential mechanisms by which EGT supplementation may target endothelial cell and macrophage processes involved in the development of vascular disease.

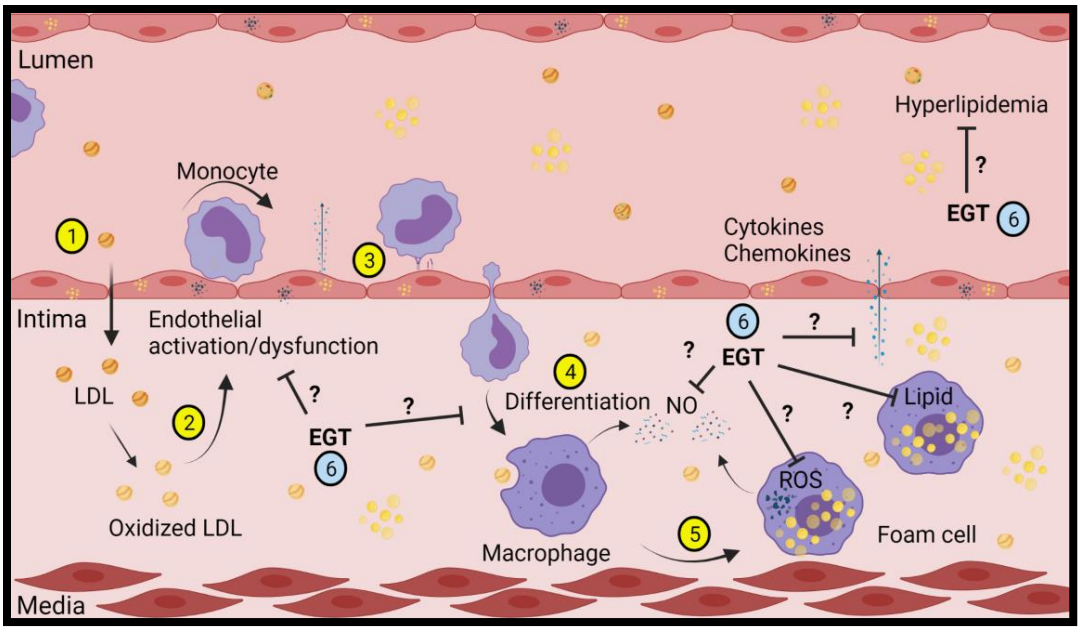


Figure 1.3 Summary of endothelial cell and macrophage responses under high lipid conditions in vascular disease and the potential protective effects of EGT

(1) During obesity and metabolic syndrome, elevated levels of circulating lipids induce endothelial cell damage which allows low density lipoprotein (LDL) particles to deposit into the intima. (2) Once in the intima, LDL particles undergo oxidation, leading to the activation of endothelial cells. These activated endothelial cells start expressing adhesion molecules at their cell surface and release pro-inflammatory cytokines and chemokines into the blood circulation. (3) In response, circulating monocytes are recruited to the vascular wall where they adhere to endothelial cells and migrate into the intima. (4) Infiltrating monocytes differentiate into macrophages, phagocytose oxidized LDL and secrete a variety of pro-inflammatory cytokines, reactive oxygen species (ROS) and nitric oxide (NO) into the extracellular matrix. This results in further recruitment of macrophages and propagation of inflammation. (5) During chronic exposure to lipids and lipoproteins such as LDL, macrophages become increasingly lipid-laden and are known as foam cells. Foam cell and lipid accumulation in the intima intensifies the inflammatory environment leading to the development and progression of atherosclerosis. (6) EGT may help protect against lipid induced oxidative stress and inflammation within the vasculature by decreasing NO and ROS accumulation, hyperlipidemia and foam cell lipid accumulation. Figure was created in BioRender.

1.5 Experimental Models chosen for Thesis

To study the key cell types involved in modifying atherosclerosis risk, human microvascular endothelial cells (HMVEC) and human monocytic THP-1 cells (differentiated into macrophages using phorbol 12,13-dibutyrate (PDB)) were chosen as *in vitro* models as they closely resemble the cells that are found within the human vasculature. As previously mentioned in section 1.2, endothelial cells and macrophages are some of the first vascular cell types to be exposed to high circulating lipids [18,19]. Endothelial cells play a critical role in vasculature homeostasis by regulating vascular permeability, vessel repair and angiogenesis [25,29]. During endothelial dysfunction, vascular permeability is disrupted, which causes the endothelium to become leaky and leads to more lipid deposition and monocyte migration into the vasculature [23]. Additionally, endothelial dysfunction disrupts the ability of the vasculature to form new blood vessels in response to vascular injury and tissue ischemia due to atherosclerosis [30]. Macrophages on the other hand are responsible for the inflammatory response to the excess lipid accumulation by producing pro-inflammatory cytokines and ROS [44]. However, chronic exposure to high lipid levels disrupts the ability of macrophages to resolve this inflammation leading to the development and progression of vascular disease [106].

To study the effects of EGT on the vasculature under a high lipid conditions *in vivo*, a diet-induced obese mouse model was chosen. Mice are one of the most cost-effective animal models in research, but one caveat is their hyperlipidemic response to high fat diets. Mouse lipoprotein profiles are significantly different from those of humans [107]. While humans transport the majority of their cholesterol in LDL, mice transport most of their cholesterol in HDL [108]. This is a result of mice lacking the cholesteryl ester transport protein (CETP), which catalyzes the transfer of cholesteryl esters from HDL to LDL and very low density lipoprotein (VLDL) [109]. Without this enzyme, mice rely on HDL to transport cholesterol and as a result, wild type mice fed a high fat diet do not develop atherosclerosis [110]. To overcome this experimental design issue, C57BL/6J LDL receptor knockout ($LDLr^{-/-}$) mice were chosen. These mice have a homozygous $Ldl^{rtm1Her}$ mutation that was created by inserting a neomycin resistance cassette into exon 4, which is predicted to encode a non-functional protein receptor that lacks a membrane spanning segment and thus

will not bind to LDL. Consequently, these mice are deficient in hepatic LDL receptors, which results in prolonged half-lives of VLDL and LDL in the circulation [107], a human-like plasma lipid profile, and the development of hyperglycemia, hyperinsulinemia, and dyslipidemia with small foam cell lesions in the aortic arch when fed a high fat diet [108,111]. In summary, $LDLr^{-/-}$ mice are a well-established research model for the study of factors that modify obesity, metabolic syndrome, and atherosclerotic risk when fed a high fat diet [112–117].

1.6 Objectives and Hypothesis

Rationale

The elevated concentrations of circulating lipids that are present in obesity and metabolic syndrome are tightly linked to the development of vascular disease [13]. Potential targets for modifying vascular disease risk in individuals who suffer from obesity and metabolic syndrome include dysfunctional endothelial cells and macrophages, and blood lipids [17]. Chronic exposure to elevated plasma lipid levels induces oxidative stress and inflammation within the vasculature leading to endothelial dysfunction and persistent pro-inflammatory activation of macrophages [25,27]. EGT may be a potential candidate to protect against vascular cell stress induced by high lipid levels as it is well known for its antioxidant and potential anti-inflammatory properties [51,52,60]. There is also some evidence that EGT may modify vascular disease risk by altering blood lipid profiles [64]. However, the effects of EGT on vascular cells under high lipid conditions, and on blood lipid and lipoprotein profiles in diet-induced obese, hyperlipidemic mice have not been studied.

Objectives

The objectives of this thesis were to determine whether EGT supplementation could modify:

1) endothelial cell and macrophage function during exposure to high fatty acid conditions *in vitro*.

2) characteristics of metabolic syndrome (blood glucose and blood lipids) as markers of atherosclerotic risk in diet-induced obese $LDLr^{-/-}$ mice.

Hypothesis

EGT supplementation will decrease endothelial cell dysfunction and macrophage inflammatory characteristics under high lipid conditions, and markers of atherosclerotic risk in diet-induced obese, hyperlipidemic mice.

Chapter 2

2 Materials and Methods

2.1 Cell Culture and Treatments

HMVEC and human monocytic THP-1 cells were chosen for *in vitro* experiments as they are considered a close representation of the cells that are found within the human vasculature. In addition, these cell types express OCTN1 transporters which are responsible for facilitating EGT entry into cells [74,75]. All cells were cultured at 37 °C and 5 % CO_2 in humidified conditions. HMVEC from normal skin tissue of female adult donors were obtained from PromoCell and cultured in Medium 199 (Gibco) supplemented with EGM-2-MV SingleQuots (Lonza). Cells were grown in 100 mm culture dishes and medium was changed every 2 to 3 days. Cells were subcultured at 80 % confluency using trypsin-Ethylenediaminetetraacetic acid (EDTA) solution (Lonza) and subcultures between 4 and 8 were used for all experiments in order to ensure that predictable endothelial cell morphology and growth behavior was maintained. Human monocytic THP-1 cells derived from the peripheral blood of a male child with acute monocytic leukemia were obtained from the American Type Culture Collection (ATCC) [118]. These cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco) supplemented with 50 μM β-mercaptoethanol (Sigma), 10 % fetal bovine serum (FBS) (Gibco), and penicillin/streptomycin solution (100 U/mL and 100 μg/mL) (Gibco). Cells were grown in T-75 and T-125 culture flasks, the medium was changed every 2 to 3 days, and cells were subcultured at 1 x 10^6 cells/mL. For experiments, THP-1 monocytes were differentiated to macrophages by exposure to PDB (300 nM; Sigma) for 5 days.

Fatty acid-supplemented medium was prepared following a modification of the procedure conducted by Listenberger et al. (2001) [119]. Briefly, separate solutions of 20 mM palmitate and oleate (Sigma) in 0.01 M NaOH were incubated at 70 °C for 30 minutes, with dropwise addition of 1 M NaOH in order to solubilize the fatty acids. Palmitate and oleate were then complexed with 30 % fatty acid-free bovine serum albumin (BSA; Sigma) at a molar ratio of 2:1 prior to the addition of medium to reach a total fatty acid concentration of 0.5 mM [120]. A mixture of palmitate plus oleate (PA/OA) at a ratio of

2:3 was prepared to reflect the ratio of saturated to unsaturated fatty acids commonly found in western diet [121]. Total fatty acid concentration of 0.5 mM was used to represent pathophysiological blood concentrations observed in human obesity and metabolic syndrome [122,123]. Growth medium supplemented with BSA alone acted as the control for fatty acid treatments. EGT (Sigma-Aldrich) (Oakville, Ca) was solubilized in cell culture grade water and added to experimental growth medium to obtain a final concentration of 0.25 mM. This concentration was chosen as it is within the range of concentrations (100 μM to 2 mM) that were found within cells and tissues [52]. The concentration of EGT that could be in the serum of the experimental growth medium is unknown but considered to be negligible. In addition, we measured cell metabolism as an indicator of cell viability at several concentrations of EGT using standard MTT [3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide] assays. For all experiments, cells were treated with 0.25 mM of EGT or vehicle control (water) in the presence of PA/OA or BSA alone (control).

2.2 Cell Viability

HMVEC and THP-1 macrophages were plated in 96 well plates, grown to approximately 80 - 90 % confluency, and treated with EGT and/or fatty acids in triplicate for 18 hours. Ten μL of MTT (5 mg/mL in PBS (phosphate buffered saline); Sigma) reagent was added to each well and allowed to sit for 3 hours, followed by the addition of 100 μL/well of MTT lysis buffer. Plates were incubated in the dark overnight at room temperature and followed by measurement of absorbance at 595 nm using a microplate spectrophotometer (SpectraMax M3).

2.3 HMVEC Tube Formation and Stability

To assess angiogenic function, HMVEC were plated on a growth factor replete Matrigel basement membrane Matrix (BD Biosciences), in 96-well plates (11,400 cells/well) and treated with EGT and/or fatty acids. Images were taken using light microscopy at 18 hours to assess tube formation and again at 24 and 42 hours to assess tube stability. Maximum tube formation occurs at 18 hours after which, endothelial tubes start to degrade [120,124–126]. Total tube length per field of view was quantified using ImageJ software for all

treatments at each time point. A tube was defined as an apparently three-dimensional, elongated structure stretching between branch points, with a width large enough along its entire length to permit the passage of an erythrocyte. Areas under the curves were generated using total tube lengths from all 3 time points to assess tube stability as previously described [120,124,127]. Three separate types of experiments were conducted for the Matrigel assay which included: no pre-incubation with EGT (normoxia, 20% O_2), an 8-hour pre-incubation with EGT (normoxia, 20% O_2), and an 8-hour pre-incubation with EGT followed by incubation in hypoxic conditions (2% O_2).

2.4 HMVEC and THP-1 NO Concentration

HMVEC and THP-1 macrophages were plated in 96 well plates, grown to approximately 80 - 90 % confluency, and treated with EGT and/or fatty acids for 18 hours. After incubation, conditioned media were collected on ice and stored at -80 °C prior to processing. To assess NO concentration, total nitrite concentrations were measured using a commercially available Total Nitric Oxide and Nitrate/Nitrite Parameter Assay Kit (R&D Systems) following the manufacturer's protocol. NO has a relatively short half-life and breaks down into nitrate and nitrite. The colorimetric assay converts nitrate into nitrite through the enzyme nitrate reductase. Total nitrite was then detected as an azo dye product of the Griess reaction at a wavelength of 540 nm (SpectraMax M3 spectrophotometer). All samples fell within the standard curve which ranged between 3.13 - 200 μM. Total nitrite concentrations from samples were normalized to total protein concentration determined using a commercially available bicinchoninic acid (BCA) assay (Thermo Scientific). Briefly, protein was extracted from cells through the addition of 0.1 M NaOH to each well followed by an incubation overnight at room temperature. Protein samples and BCA reagents were added to 96 well plates and subsequently incubated at 37 °C for 30 minutes before being read at a wavelength of 550 nm (SpectraMax M3 spectrophotometer).

2.5 THP-1 Monocyte to Macrophage Differentiation

THP-1 monocytes were plated in 100 mm culture dishes (1.6×10^7 cells/dish), differentiated using PDB (300 nM) as previously described [128], and treated with EGT and/or fatty acids for 72 hours. At 0 (undifferentiated, untreated THP-1 monocytes) and 72

hour time points, images were taken using light microscopy and RNA was collected using the RNeasy Mini Kit (QIAGEN) following the manufacturer's protocol. RNA concentration and purity were measured using the NanoDrop spectrophotometer (Thermo Scientific). Two ug of RNA was diluted in 9 ul of nuclease-free water before RNA was converted to cDNA by reverse transcription using a High Capacity RNA-to-cDNA kit (Applied Biosystems) following the manufacturer's protocol.

For real-time quantitative polymerase chain reaction (RT-qPCR), reagents and TaqMan primers including glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), cluster of differentiation 14 (*CD14*), myeloblastosis (*MYB*), *IL-1β* and chemokine ligand 20 (*CCL20*) were used (Applied Biosystems). In nuclease free tubes, RNase free water, 2X TaqMan gene expression master mix, cDNA sample and TaqMan primer were combined, vortexed and transferred (20 µl) into a 384-well reaction plate in duplicate. Plates were sealed, mixed, briefly centrifuged, and loaded into the ViiA 7 qRT-PCR system (Applied Biosystems). Reactions were run for 40 cycles with a 15 second denaturation step at 95 °C followed by a 1 minute anneal/extension step at 60 °C. Cycle threshold (Ct) values were used to calculate ΔΔCt values and expression fold changes ($2^{-\Delta\Delta Ct}$) for each sample. *GAPDH* was used as the endogenous control for all reactions. Expression levels were normalized to those of undifferentiated, untreated THP-1 monocytes.

2.6 THP-1 Macrophage ROS Accumulation

THP-1 macrophages were plated in 6 well plates, grown to approximately 80 - 90 % confluency, and treated with EGT and/or fatty acids for 18 hours. After incubation, cells were stained with 5 µM CellROX® Deep Red Reagent (Invitrogen™) for 30 minutes at 37 °C. Cells were then fixed with 4 % paraformaldehyde and nuclei were stained with Hoechst 33342 Ready Flow™ Reagent (Invitrogen™) for 30 minutes at room temperature. Images were taken using the Olympus 1X81 fluorescence microscope at excitation and emission wavelengths of 640 and 665 nm (deep red), respectively. Deep red signal area was measured using ImageJ and normalized to the number of nuclei stained with Hoechst reagent.

2.7 THP-1 Macrophage Cytokine Secretion

THP-1 macrophages were plated in 6 well plates, grown to approximately 80 - 90 % confluency, and treated with EGT and/or fatty acids for 24 and 48 hours. After incubation, conditioned media were collected on ice and stored at -80 °C prior to processing. IL-1β and IL-6 concentrations were measured using a human IL-1β enzyme-linked immunosorbent assay (ELISA) kit II (BD Biosciences) and an IL-6 high sensitivity human ELISA kit (Abcam) following the manufacturer's protocol. All samples fell within the standard curves for IL-1β and IL-6 which ranged between 1.953 - 125 pg/mL and 1.56 - 50 pg/mL, respectively. Cytokine concentrations were normalized to total protein concentrations determined through the previously described BCA assay.

2.8 THP-1 Macrophage Lipid Accumulation

THP-1 monocytes were plated in 6 well plates (4×10^6 cells/well), differentiated into macrophages and subsequently treated with EGT and/or fatty acids in duplicate for 18 hours. After incubation, cells were washed twice with 2 mg/mL BSA dissolved in PBS followed by 3 washes with PBS alone. To extract lipids, a hexane and isopropanol (3:2) mixture was added to each well for 30 minutes before transferring to glass tubes. This process was repeated once more before fully evaporating the solution to dryness under nitrogen gas. Equal volumes of chloroform and 1% Triton X-100 in chloroform were added to each sample and incubated overnight at room temperature. The next day, samples were evaporated to dryness under nitrogen gas before deionized water was added. Samples were then mixed and placed in a water bath at 37 °C for 20 minutes. Triglyceride (Roche), total cholesterol (Wako Diagnostics) and free cholesterol (Wako Diagnostics) were measured using commercially available enzymatic, colorimetric assays following the manufacturer's protocol. All samples fell within the standard curves for triglyceride, total cholesterol and free cholesterol which ranged between 0.5 – 20 μg. Cholesteryl ester was calculated by subtracting free cholesterol from total cholesterol. Lipid concentrations were normalized to total protein concentrations determined through the previously mentioned BCA assay.

2.9 Mouse study

All mouse studies were approved by the Animal Use Subcommittee at Western University (protocol 2020-073) and were designed according to the Canadian Council on Animal Care guidelines. Six-week-old, male B6.129S7-Ldlr$^{tm1Her/J}$ mice (Jackson Laboratories, stock #002207) were fed western diet (TD.88137, Envigo) (42 % kcal from fat, 0.2 % kcal from cholesterol) (n=20) *ad libitum* for a total of 16 weeks. This mouse strain is on a C57BL/6J background and is LDL receptor deficient (LDLr$^{-/-}$). Wild type mice and chow diets were not used for two reasons: 1) as described in section 1.5, feeding LDLr$^{-/-}$ mice western diet for 16 weeks is a well-established protocol that induces obesity, metabolic disease and atherosclerotic risk [112–117], and 2) the objective of this study was to evaluate the effect of EGT on parameters of metabolic syndrome that are markers of atherosclerotic risk.

Mice were housed in pairs for 13 weeks, after which they were put into individual cages and randomly assigned to two groups to continue western diet supplemented with either distilled, deionized water (control) or 40 mg of EGT per kilogram body weight per day. This dosage was chosen based on previous studies indicating that this dose significantly increased EGT concentration in a variety of tissues including the heart, liver, and intestines after 1 and 4 weeks of daily oral gavage [79]. Western diet supplemented with EGT (0.05%) was made by solubilizing EGT in distilled, deionized water, blending with western diet using a food processor and subsequently repacking by hand into food pellets. Body weight and food intake were measured once per week prior to treatment, after which food intake was measured twice per week in addition to weekly measurements of body weights during the supplementation period. After a total of 16 weeks (13 weeks of western diet feeding, plus 3 weeks of supplementation with EGT), mice were fasted for 4 hours prior to sacrifice. Blood glucose concentrations were measured immediately prior to sacrifice using a handheld glucometer (Bayer).

Upon sacrifice, body weight was recorded, blood was collected by cardiac puncture and liver and adipose tissue were harvested and weighed. In addition, the heart, aortic sinus, jejunum, ileum and pancreas were harvested for future studies. Plasma triglyceride and total cholesterol were determined using enzymatic, colorimetric assays following the manufacturer's protocol. All samples fell within the standard curve for triglyceride and

cholesterol which ranged between 0.065 – 3.09 mM and 0.24 – 11.7 mM, respectively. Plasma insulin was measured using an ultra-sensitive mouse insulin ELISA (Crystal Chem) and all samples fell within the standard curve which ranged between 0.1 - 12.8 ng/mL. Liver lipids were extracted by the Folch method [129] and liver triglyceride, total cholesterol and free cholesterol were measured using enzymatic, colorimetric assays following the manufacturer's protocol. All samples fell within the standard curve which ranged between 0.5 - 20 μg. Cholesteryl ester was calculated by subtracting free cholesterol from total cholesterol.

Fast protein liquid chromatography (FPLC) was used to determine the distribution of plasma lipoproteins. Fresh EDTA plasma (50 μL) was separated by FPLC using an AKTA purifier and a Superose 6 column (GE Healthcare Life Sciences). A constant flow rate of 0.4 mL/min was used to collect 40 fractions (750 μL/fraction). Triglyceride and total cholesterol were measured in FPLC fractions using enzymatic, colorimetric assays. All samples fell within the standard curve for triglyceride and cholesterol which ranged between 5.7 - 365 μg/mL and 9.4 - 604 μg/mL, respectively. Based on protein concentrations that were monitored at a wavelength of 280 nm, fractions 5 - 10 were determined to represent triglyceride rich lipoproteins (TRL), fractions 11 - 18 represented LDL, and fractions 22 - 28 represented HDL. Line graphs were generated for triglyceride and cholesterol measurements for each fraction, and areas under the curves were calculated for each lipoprotein type (TRL, LDL, and HDL) based on its corresponding fractions.

2.10 Statistical Analysis

All statistical analyses and generation of graphs were performed using GraphPad Prism 6. Data for the *in vitro* assays was analyzed using a one-way ANOVA followed by a Sidak post-hoc test to identify differences between BSA and PA/OA, BSA and BSA + EGT, and PA/OA and PA/OA + EGT. In addition, a one-way ANOVA followed by a Dunnett's post-hoc test was performed on HMVEC viability data to compare different concentrations of EGT to the vehicle control. Data for *in vivo* measurements were assessed using unpaired t-tests. Differences in means were considered statistically significant at $p < 0.05$.

Chapter 3

3 Results

3.1 EGT effects on HMVEC viability under high fatty acid conditions

To determine the concentration of EGT to be used in HMVEC experiments under high fatty acid conditions, we measured cell metabolism as an indicator of cell viability at several concentrations of EGT using an MTT assay. Low millimolar concentrations were chosen as a starting point because previous studies have shown protective effects against oxidative stress in endothelial cells at this concentration range [74,84,94]. Cells treated with 0.25, 0.50 and 0.75 mM of EGT showed a significant decrease in cell viability compared to the control under BSA conditions (Figure 3.1A). However, under high PA/OA conditions, only 0.50 and 0.75 mM of EGT showed a significant decrease in cell viability (Figure 3.1B). Therefore, 0.25 mM EGT was used for all subsequent *in vitro* experiments despite the decrease in cell viability under BSA conditions. The decrease in cell viability may be a result of the ROS scavenging abilities of EGT that could alter cell metabolism which is being measured by the MTT assay.

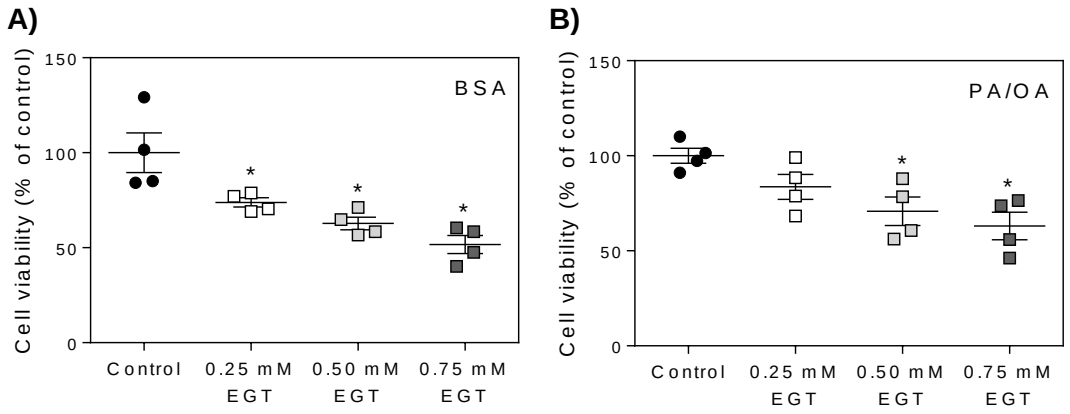


Figure 3.1 EGT effects on HMVEC viability under high fatty acid conditions

HMVEC were plated in a 96 well plate and treated with or without 0.25 mM ergothioneine (EGT) in the (A) absence or (B) presence of 0.5 mM palmitate plus oleate (PA/OA; 2:3 ratio) complexed to bovine serum albumin (BSA) in triplicate for 18 hours. Cells were then incubated with MTT reagent for 3 hours followed by the addition of MTT lysis buffer. Plates were incubated in the dark overnight at room temperature and absorbance was quantified spectrophotometrically at a wavelength of 595 nm the next day. * $p<0.05$ versus control.

3.2 EGT does not influence HMVEC tube formation and stability under high fatty acid conditions

Endothelial cells are the first to sustain damage during the development of vascular disease under high lipid conditions [23]. Therefore, I was interested in the effects of EGT on endothelial cell function. Angiogenesis is an important indicator of endothelial cell function as it plays a crucial role in the response to vascular injury and tissue ischemia [30]. Matrigel assays assess the ability of endothelial cells to migrate and proliferate to form tubular networks, which are essential for vascular regeneration and repair in response to injury [130,131]. Therefore, the effects of EGT on HMVEC tube formation and stability were investigated in three separate experiments including no pre-incubation with EGT (Figure 3.2), an 8-hour pre-incubation with EGT (Figure 3.3) and an 8-hour pre-incubation with EGT before incubation in hypoxic conditions (Figure 3.4). In all three experiments, HMVEC tube formation was not significantly affected by high concentrations of PA/OA after 18 hours of exposure. Moreover, EGT did not have an effect in either BSA or PA/OA conditions (Figure 3.2B, Figure 3.3B and Figure 3.4B). HMVEC tube stability was also not significantly affected by high concentrations of PA/OA after 24 and 42 hours of exposure. In addition, EGT supplementation did not have an effect in either BSA and PA/OA conditions (Figure 3.2C, D, Figure 3.3C, D, and Figure 3.4C, D).

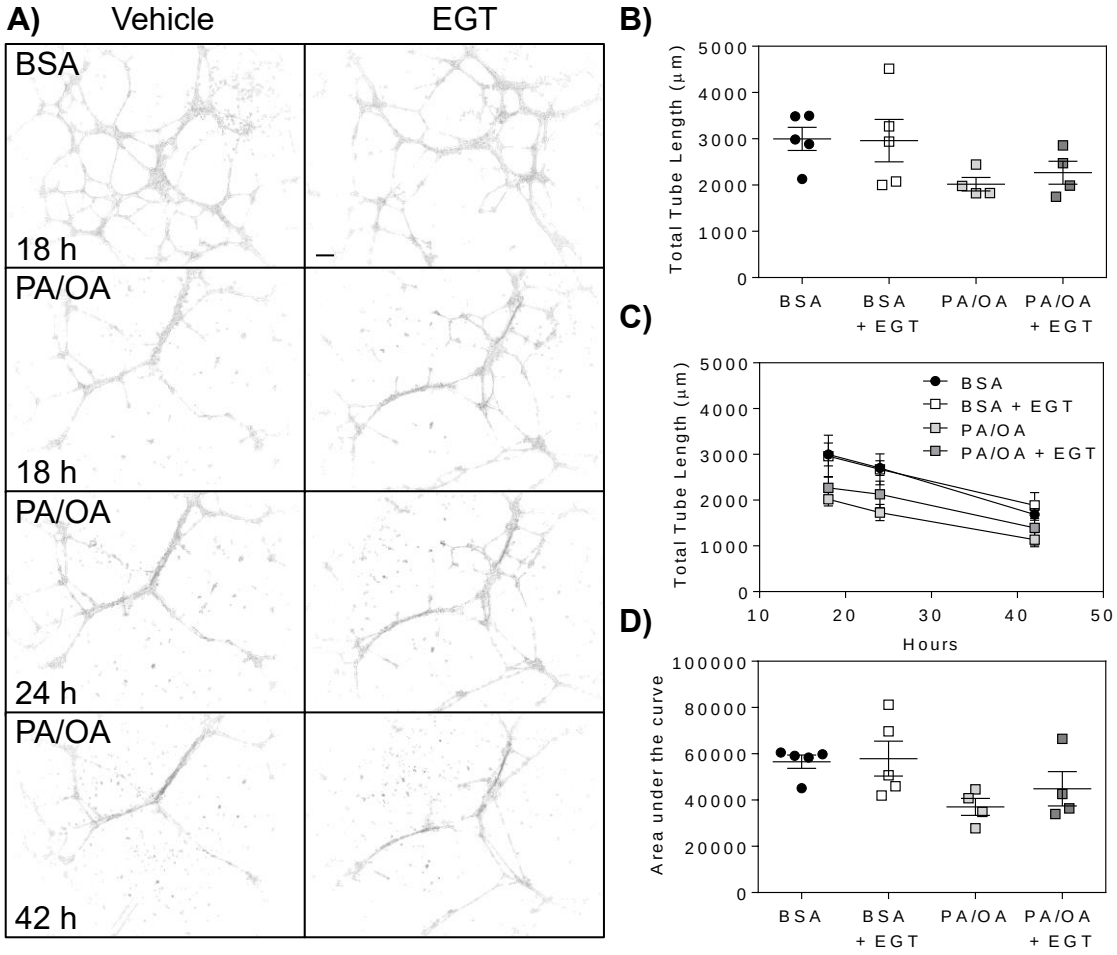


Figure 3.2 EGT does not influence HMVEC tube formation and stability under high fatty acid conditions

HMVEC were plated on growth factor replete Matrigel and treated with or without 0.25 mM EGT in the presence or absence of 0.5 mM PA/OA (2:3 ratio) complexed to BSA. (A) Cells were imaged at 18, 24 and 42-h time points using light microscopy and (B, C) total tube length per field of view was quantified using ImageJ. (D) Areas under the curves were calculated from data in (C). Data are means ± SEM, n=4-5. Scale bar = 200 μm.

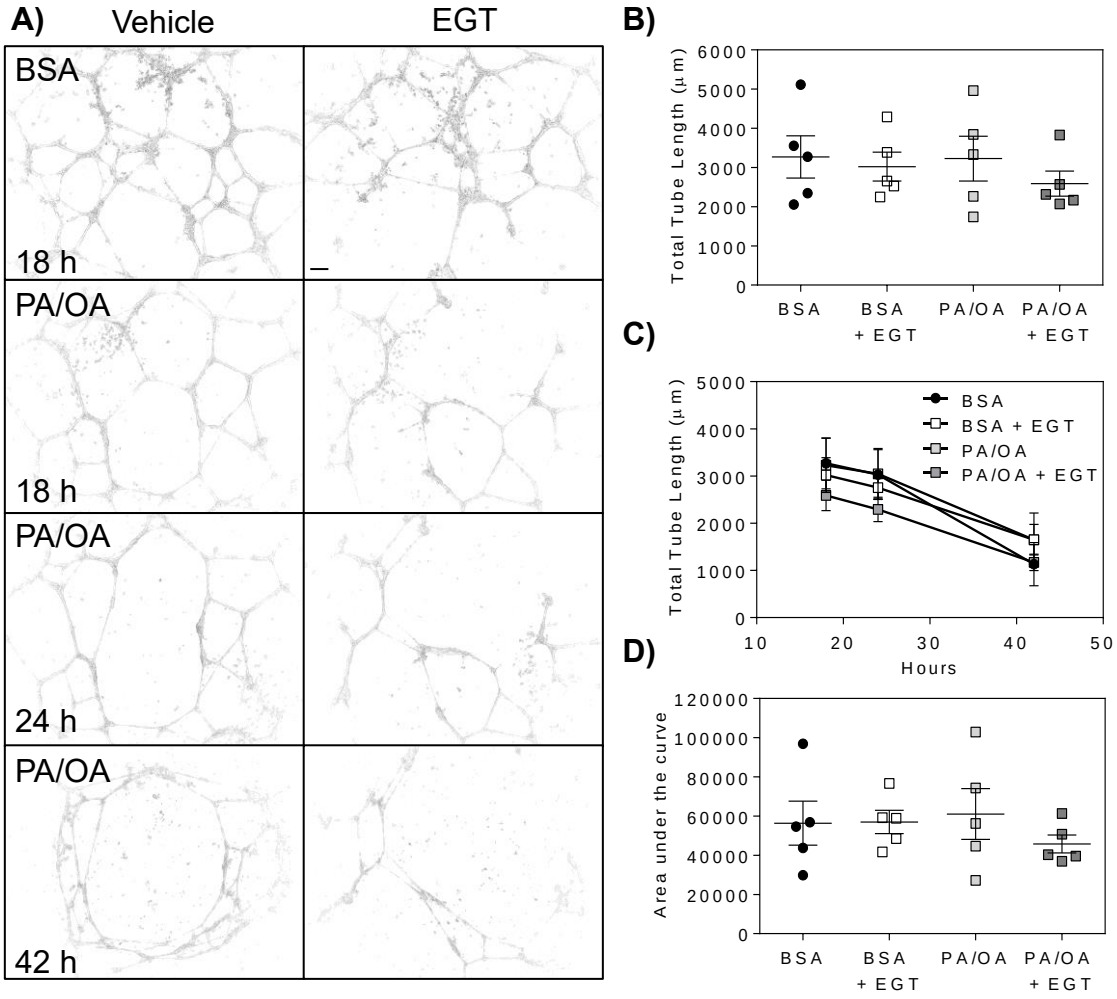


Figure 3.3 Pre-incubation with EGT does not influence HMVEC tube formation and stability under high fatty acid conditions

HMVEC were pre-incubated with or without 0.25 mM EGT for 8 hours before being plated on growth factor replete Matrigel. Cells were then treated with or without 0.25 mM EGT in the presence or absence of 0.5 mM PA/OA (2:3 ratio) complexed to BSA. (A) Cells were imaged at 18, 24 and 42-h time points using light microscopy and (B, C) total tube length per field of view was quantified using ImageJ. (D) Areas under the curves were calculated from data in (C). Data are means ± SEM, n=5. Scale bar = 200 µm.

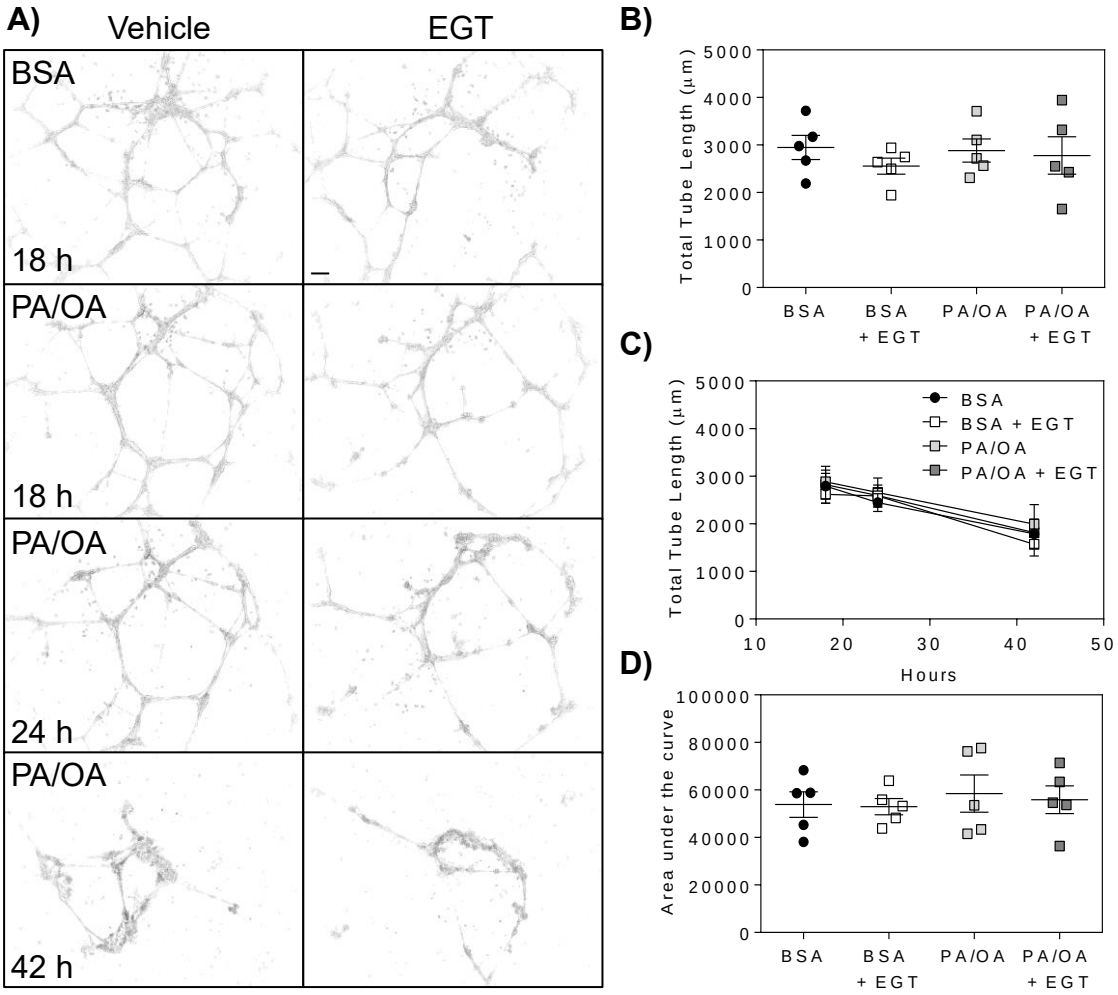


Figure 3.4 Pre-incubation with EGT does not influence HMVEC tube formation and stability under combined high fatty acid and hypoxic conditions

HMVEC were pre-incubated with or without 0.25 mM EGT for 8 hours before being plated on growth factor replete Matrigel. Cells were then treated with or without 0.25 mM EGT in the presence or absence of 0.5 mM PA/OA (2:3 ratio) complexed to BSA. Cells were incubated in hypoxic conditions (2% O_2) for a total of 42 hours. (A) Images were taken at 18, 24 and 42-h time points using light microscopy and (B, C) total tube length per field of view was quantified using ImageJ. (D) Areas under the curves were calculated from data in (C). Data are means ± SEM, n=5. Scale bar = 200 μm.

3.3 EGT reduces HMVEC NO concentration

NO plays an important role in endothelial cell function and has many protective effects in vascular disease as described in section 1.2. Decreased NO bioavailability is a hallmark for endothelial dysfunction [35]. Therefore, the effects of EGT on NO concentration under high fatty acid conditions were assessed. In two separate experiments; no pre-incubation with EGT followed by incubation in the absence or presence of EGT with BSA or PA/OA (Figure 3.5A), or 8-hour pre-incubation with EGT followed by the same conditions (Figure 3.5B), HMVEC NO concentration was not affected after 18 hours of exposure to high PA/OA (Figure 3.5A, B). However, cells treated with EGT exhibited significantly decreased NO concentration in both BSA and PA/OA conditions (Fig 3.5A, B).

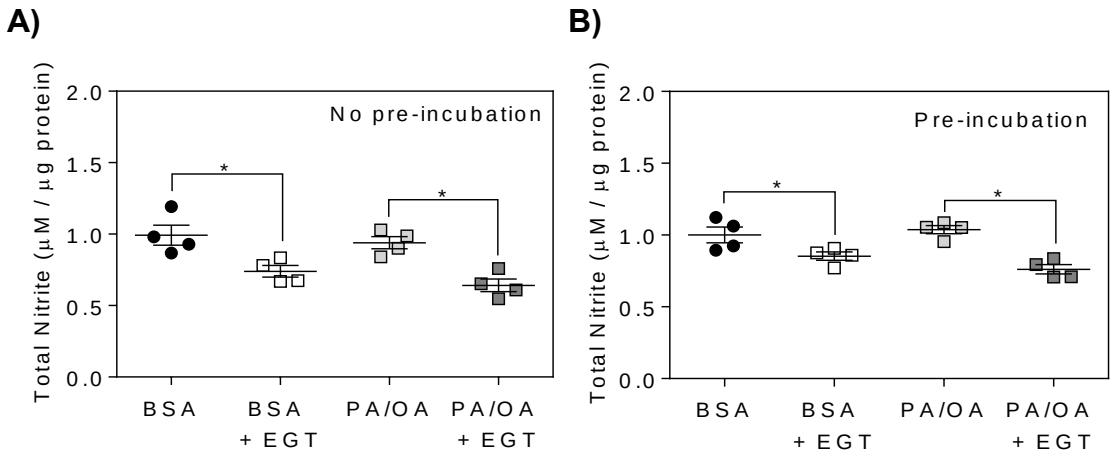


Figure 3.5 EGT decreases HMVEC NO concentration

HMVEC were incubated in the absence (A) or presence (B) of 0.25 mM EGT for 8 hours, followed by treatment with 0.25 mM EGT and 0.5 mM PA/OA (2:3 ratio) complexed to BSA for 18 hours, as indicated. After incubation, conditioned media were collected, and total nitrite concentrations were measured using a commercially available Total Nitric Oxide and Nitrate/Nitrite Parameter Assay Kit. Data are means ± SEM, n=4. *$p<0.05$.

3.4 EGT does not influence monocyte and macrophage viability under high fatty acid conditions

Endothelial cells and macrophages work closely together within the vasculature and therefore, it was appropriate to use the same concentration of EGT (0.25 mM) for experiments with THP-1 monocytes and macrophages. To assess whether 0.25 mM EGT had no effect on monocyte and macrophage viability, MTT assays were conducted. In the presence of high PA/OA, monocyte (Figure 3.6A) and macrophage (Figure 3.6B) viability were not affected after 18 hours of exposure. Moreover, 0.25 mM EGT did not influence monocyte and macrophage viability in both BSA and PA/OA conditions.

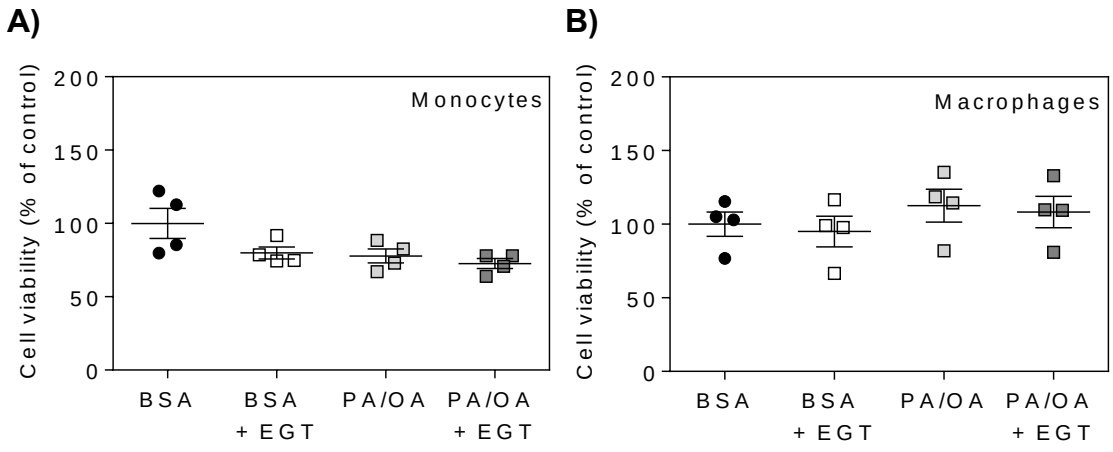


Figure 3.6 EGT does not influence monocyte and macrophage viability under high fatty acid conditions

THP-1 (A) monocytes and (B) macrophages were plated in a 96 well plate and treated with or without 0.25 mM EGT in the presence or absence of 0.5 mM PA/OA (2:3 ratio) complexed to BSA in triplicate for 18 hours. Cells were then incubated with MTT reagent for 3 hours followed by the addition of MTT lysis buffer. Plates were incubated in the dark overnight at room temperature and absorbance was quantified spectrophotometrically at a wavelength of 595 nm the next day. Data are means ± SEM, n=4.

3.5 EGT does not influence monocyte to macrophage differentiation under high fatty acid conditions

Monocyte to macrophage differentiation is one of the first steps in promoting inflammation within the vasculature under high lipid conditions, as discussed in section 1.2. When assessing morphological changes during monocyte to macrophage differentiation, lipid droplet accumulation was more prominent in cells treated with high PA/OA compared to BSA control. However, there were no visual differences in cell size or shape when cells were treated with EGT in both BSA and PA/OA conditions (Figure 3.7A). At the mRNA level, cells treated with PA/OA showed decreased gene expression of monocyte markers *CD14* and *MYB* (Figure 3.7B, C) but no differences were shown when assessing macrophage markers *IL-1β* and *CCL20* (Figure 3.7D, E). Moreover, EGT had no effect on gene expression of either monocyte (Figure 3.7B) or macrophage (Figure 3.7D, E) markers except for a small but significant decrease in *MYB* expression in BSA control conditions (Figure 3.7C).

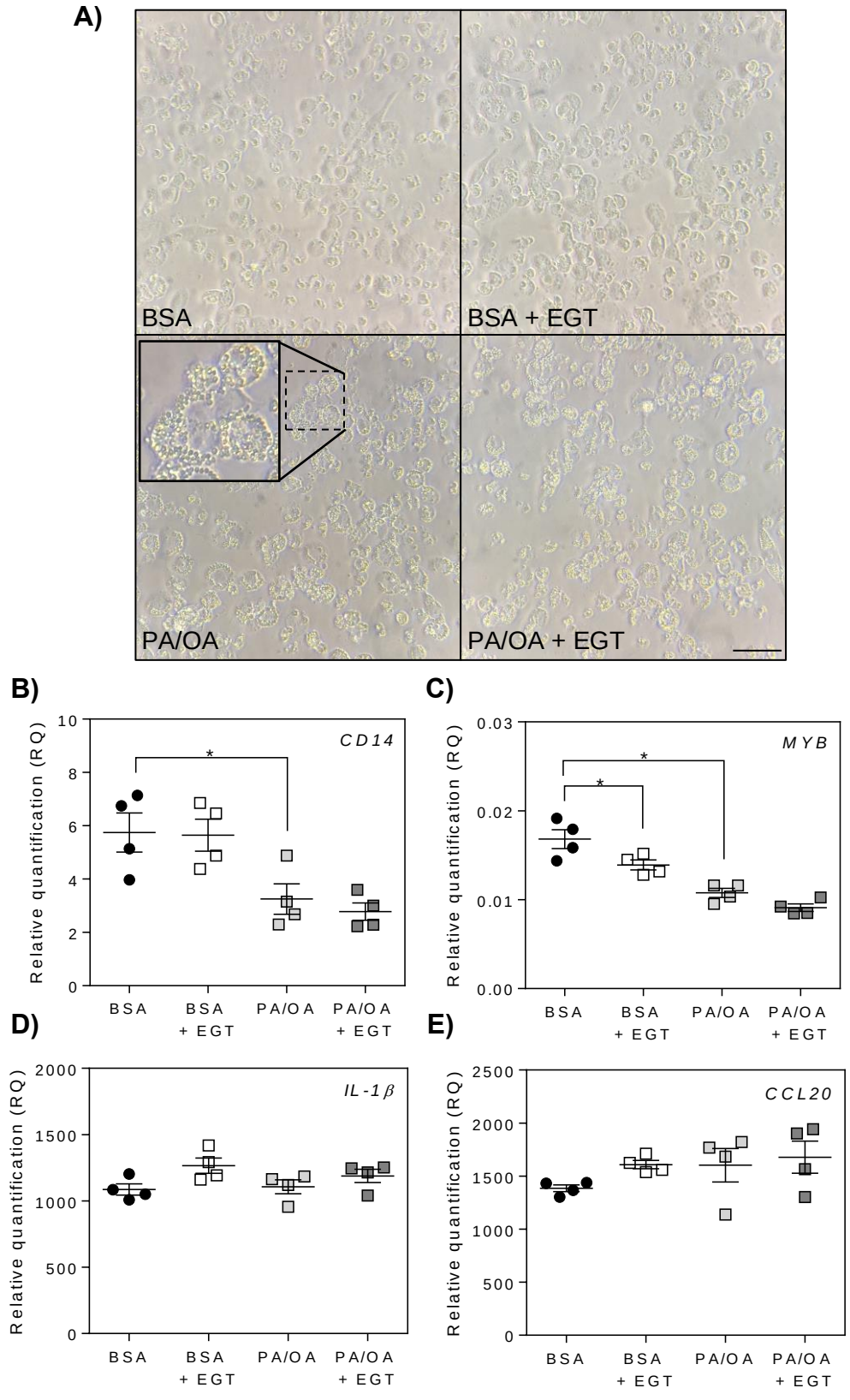

A)
BSA
BSA + EGT
PA/OA
PA/OA + EGT
B)
CD14
Relative quantification (RQ)
BSA
BSA + EGT
PA/OA
PA/OA + EGT
C)
MYB
D)
IL-1β
E)
CCL20

Figure 3.7 EGT does not influence monocyte to macrophage differentiation under high fatty conditions

THP-1 monocytes were treated with phorbol dibutyrate (PDB) and with or without 0.25 mM EGT in the presence or absence of 0.5 mM PA/OA (2:3 ratio) complexed to BSA for 72 hours. (A) Bright field images were taken and relative mRNA of (B) *CD14*, (C) *MYB*, (D) *CCL20* and (E) *IL-1β* in differentiating THP-1 cells were determined at 72 hours. Values were normalized to mRNA levels of undifferentiated, untreated THP-1 monocytes. Data are means ± SEM, n=4. *$p<0.05$. Scale bar = 50 μm.

3.6 EGT does not influence macrophage NO concentration under high fatty acid conditions

The observation that EGT decreased HMVEC NO concentration, raised the possibility that EGT could alter macrophage NO concentration. NO is produced in high concentrations by macrophages to modulate inflammatory and immune processes. In the presence of high PA/OA, macrophage NO concentration was not affected after 18 hours of exposure. Treatment of macrophages with EGT did not significantly influence NO concentration under either BSA or PA/OA conditions (Figure 3.8), although a similar magnitude of reduction ($p = 0.36$) was observed in both HMVEC (Figure 3.5A) and macrophages treated with EGT under basal (BSA) conditions (Figure 3.8).

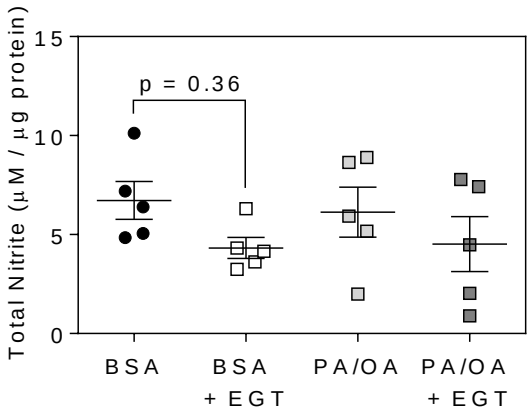


Figure 3.8 EGT does not influence macrophage NO concentration under high fatty acid conditions

THP-1 macrophages were treated with or without 0.25 mM EGT in the presence or absence of 0.5 mM PA/OA (2:3 ratio) complexed to BSA for 18 hours. After incubation, conditioned media were collected, and total nitrite concentrations were measured using a commercially available Total Nitric Oxide and Nitrate/Nitrite Parameter Assay Kit. Data are means ± SEM, n=5.

3.7 EGT reduces cytosolic ROS accumulation in macrophages

ROS are involved in many aspects of vascular disease, including promotion of inflammation, LDL oxidation, and peroxynitrite formation, as discussed in section 1.2 [21]. Given the known antioxidant activity of EGT, the ability of EGT to modulate ROS in macrophages was tested. In the presence of high PA/OA, cytosolic ROS were not affected after 18 hours of exposure. This was expected as high PA/OA conditions are considered to be lipid loading rather than inducing high oxidative stress. Interestingly, macrophages treated with EGT exhibited significantly decreased ROS accumulation in both BSA and PA/OA conditions (Figure 3.9A, C, D).

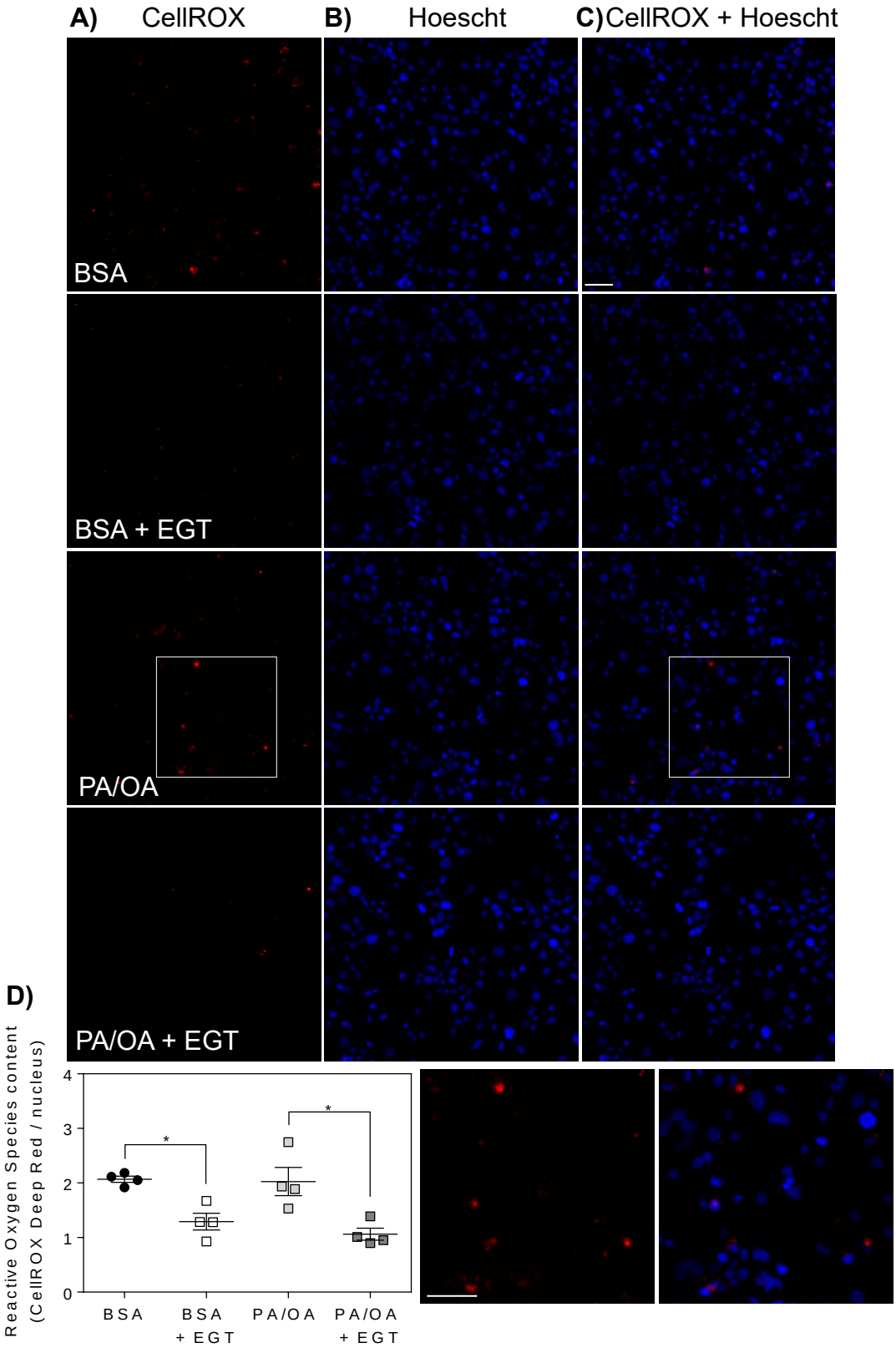

A)
CellROX
B)
Hoescht
C)
CellROX + Hoescht
BSA
BSA + EGT
PA/OA
PA/OA + EGT
D)
Reactive Oxygen Species content
(CellROX Deep Red / nucleus)
0
1
2
3
4
*
*
BSA
BSA
+ EGT
PA/OA
PA/OA
+ EGT

Figure 3.9 EGT reduces cytosolic ROS accumulation in macrophages

THP-1 macrophages were treated with or without 0.25 mM EGT in the presence or absence of 0.5 mM PA/OA (2:3 ratio) complexed to BSA for 18 hours. Cells were then stained with (A) CellROX Deep Red Reagent to visualize ROS and (B) Hoechst to visualize nuclei, and imaged on a fluorescence microscope to obtain signals for both fluorophores (C). Total red area was measured, nuclei were counted, and ROS content was expressed as the CellROX Deep Red area per nuclei (D). Scale bar = 50 μm. Data are means ± SEM, n=4. *$p<0.05$

3.8 EGT promotes macrophage IL-6 secretion but does not influence IL-1β secretion under high fatty acid conditions

Pro-inflammatory cytokines secreted by macrophages are responsible for recruiting monocytes to the vasculature to sustain or elevate vascular inflammation [28], and EGT has been shown to decrease inflammatory cytokine production in various cell types [90–92]. Therefore, the effects of EGT on the accumulation of two pro-inflammatory interleukins, IL-6 and IL-1β, were investigated. Exposure of macrophages for 24 and 48 hours to high PA/OA increased IL-1β secretion, but did not affect IL-6 secretion (Figure 3.10). Interestingly, when cells were treated with EGT in PA/OA conditions, IL-6 secretion was significantly increased (Figure 3.10C, D) while no changes in IL-1β secretion were observed (Figure 3.10A, B).

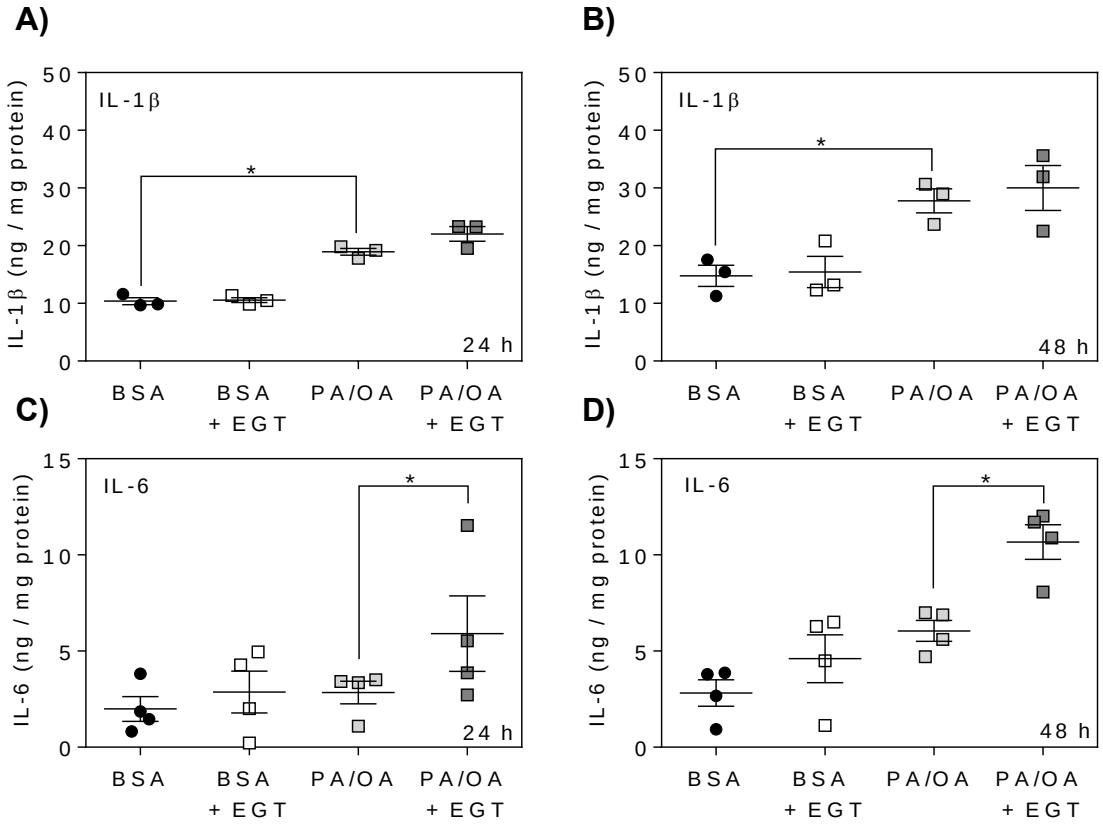


Figure 3.10 EGT promotes macrophage IL-6 secretion but does not influence IL-1β secretion under high fatty acid conditions

THP-1 macrophages were treated with or without 0.25 mM EGT in the presence or absence of 0.5 mM PA/OA (2:3 ratio) complexed to BSA for (A, C) 24 and (B, D) 48 hours. After incubation, conditioned media were collected and inflammatory cytokines, (A, B) IL-1β and (C, D) IL-6, were measured using ELISA. Data are means ± SEM, n=3-4. *$p<0.05$

3.9 EGT does not influence lipid accumulation in macrophages under high fatty acid conditions

Chronic exposure to lipids within the vasculature results in the formation of lipid-laden macrophages, known as foam cells, which secrete ROS and pro-inflammatory cytokines to further promote inflammation [27]. To further assess the potential of EGT to modulate macrophage processes involved in vascular inflammation, its effects on macrophage foam cell formation were assessed. Exposure of macrophages to high PA/OA for 18 hours resulted in significant cellular accumulation of triglyceride (Figure 3.11A) but not cholesterol (Figure 3.11B, C, D). Treatment with EGT did not affect cellular triglyceride (Figure 3.11A) or cholesterol (Figure 3.11B, C, D) concentrations.

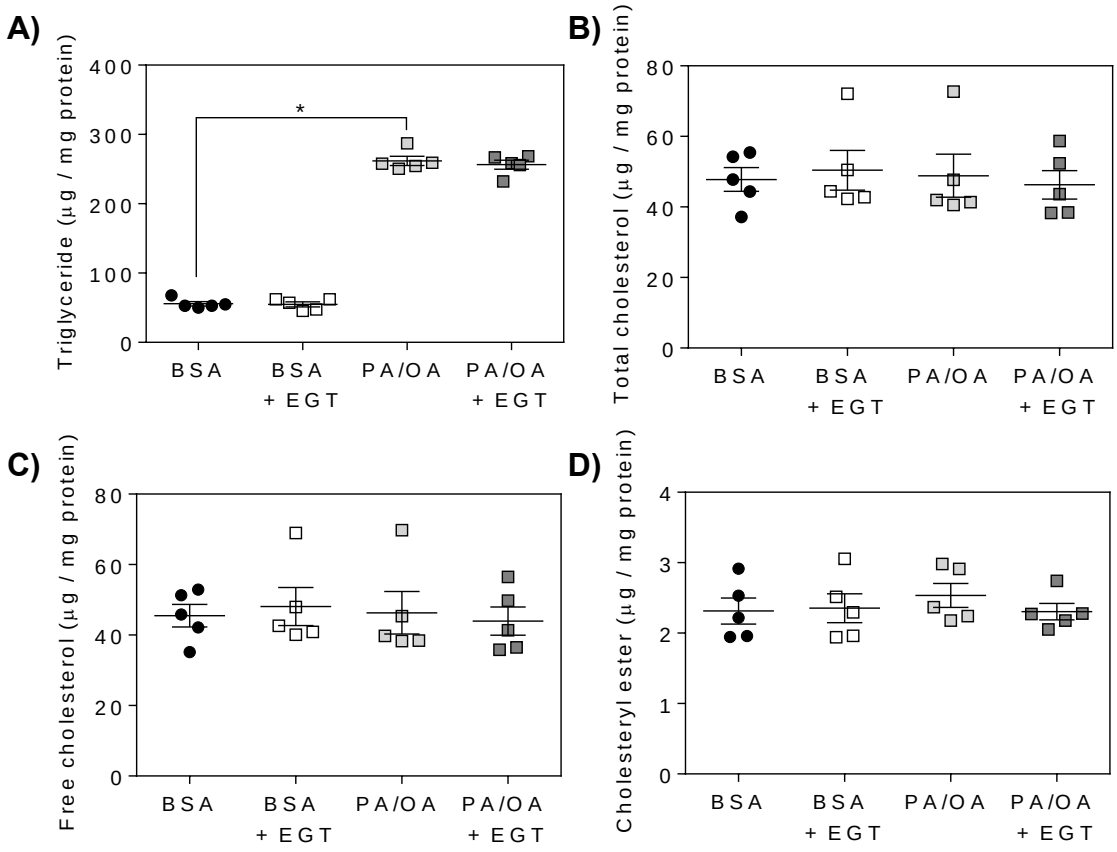


Figure 3.11 EGT does not influence lipid accumulation in macrophage under high fatty acid conditions

THP-1 macrophages were treated with or without 0.25 mM EGT in the presence or absence of 0.5 mM PA/OA (2:3 ratio) complexed to BSA for 18 hours. Lipids were extracted and (A) triglyceride, and (B, C, D) cholesterol concentrations were measured using commercially available enzymatic, colorimetric assays. Data are means ± SEM, n=5. *$p<0.05$

3.10 Assessment of parameters of metabolic syndrome in diet-induced obese, hyperlipidemic mice

Currently, there are a limited number of *in vivo* studies reporting the effects of dietary EGT supplementation in rodent models of disease. To our knowledge, there are no studies on the effects of EGT-supplemented diets on characteristics of metabolic syndrome and risk factors for vascular disease in diet-induced obese, hyperlipidemic mice. To determine the effect of dietary EGT supplementation on characteristics of metabolic syndrome that are considered risk factors for atherosclerosis and vascular disease, male C57BL/6J LDLr$^{-/-}$ mice were fed western diet (42 % kcal from fat, 0.2 % kcal from cholesterol) for a total of 16 weeks *ad libitum* to induce increased adiposity, hyperlipidemia, hyperinsulinemia, mild hyperglycemia, and hepatic steatosis (Table 3.1). When comparing mice that received western diet supplemented with EGT to control mice fed western diet alone, no significant differences were observed in body weight (Figure 3.12A) or food consumption (Figure 3.12B) over the 3-week EGT supplementation period. After the first week of EGT supplementation (week 14), food consumption was increased which was most likely a result of less competition for food as these mice were individually caged at the start of the supplementation period as described in section 2.9. Moreover, there were no differences in epididymal fat weight, liver triglyceride and cholesterol, plasma triglyceride, cholesterol and insulin, and blood glucose levels (Table 3.1).

Table 3.1 Parameters of metabolic syndrome in C57BL/6J LDLr$^{-/-}$ mice

Treatment	Western	Western + EGT
Initial body weight (g)	21.12 ± 0.34	21.45 ± 0.30
Body weight at week 13 (g)	36.85 ± 0.82	36.53 ± 0.97
Body weight at sacrifice (g)	40.77 ± 0.90	40.31 ± 0.96
Food Intake (kcal/day)	13.94 ± 0.30	14.04 ± 0.41
Epidydimal fat weight (g)	2.29 ± 0.09	2.28 ± 0.10
Liver weight (g)	2.47 ± 0.15	2.30 ± 0.14
Liver triglycerides (mg/g)	153.80 ± 10.77	133.80 ± 10.35
Liver total cholesterol (mg/g)	16.19 ± 0.72	14.92 ± 1.22
Liver cholesteryl ester (mg/g)	12.30 ± 0.73	11.11 ± 1.04
Liver free cholesterol (mg/g)	3.90 ± 0.12	3.80 ± 0.23
Plasma triglycerides (mmol/L)	9.54 ± 0.94	9.93 ± 1.00
Plasma cholesterol (mmol/L)	45.08 ± 2.60	47.80 ± 3.51
Blood glucose (mmol/L)	7.87 ± 0.29	8.10 ± 0.22
Plasma insulin (ng/ml)	3.70 ± 0.72	3.64 ± 0.38

Six-week-old, male C57BL/6J mice with a low density lipoprotein receptor knockout (LDLr$^{-/-}$) were fed on western diet (42 % kcal from fat, 0.2 % kcal from cholesterol) *ad libitum* for a total of 16 weeks. On week 13, mice were put into individual cages and randomly assigned to two groups to continue western diet supplemented with or without EGT (40 mg/kg/day) for 3 weeks. Body weight and food intake were measured once per week prior to treatment, after which food intake was measured twice per week in addition to the weekly body weight measurements during the supplementation period. Prior to sacrifice, body weight, food intake and fasting blood glucose were measured. All other parameters were measured post-mortem. Data are means ± SEM, n = 10.

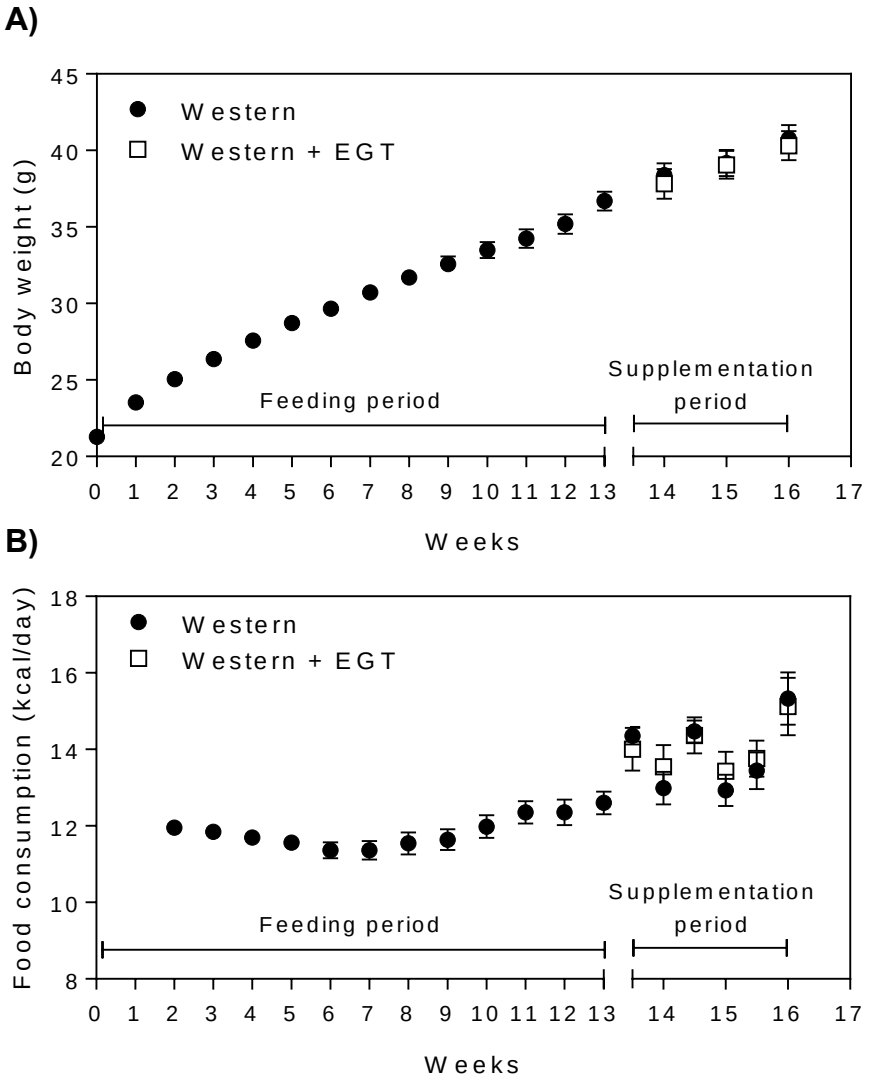


Figure 3.12 Body weight and food consumption of C57BL/6J LDLr$^{-/-}$ mice

Six-week-old, male C57BL/6J LDLr$^{-/-}$ mice were fed western diet (42 % kcal from fat, 0.2 % kcal from cholesterol) *ad libitum* for a total of 16 weeks. On week 13, mice were put into individual cages and randomly assigned to two groups to continue western diet supplemented with or without EGT (40 mg/kg/day) for 3 weeks. (A) Body weight and (B) food intake were measured once per week prior to treatment, after which food intake was measured twice per week in addition to the weekly body weight measurements during supplementation period. Data are means ± SEM. n=10.

3.11 EGT supplementation does not affect lipoprotein profiles of diet-induced obese, hyperlipidemic mice

As previously mentioned in section 1.5, LDLr$^{-/-}$ mice were used for this study as they develop characteristics of metabolic syndrome and a hyperlipidemic lipoprotein profile similar to humans when fed high fat, cholesterol-containing diets. This allowed for determination of the effects of EGT supplementation on plasma lipoprotein profiles which are known risk factors for vascular disease and atherosclerosis. In the current study, mice showed high concentrations of cholesterol in TRL and LDL plasma fractions, as expected for this model of diet-induced hyperlipidemia (Figure 3.13B). However, mice that received western diet supplemented with EGT showed no significant difference in triglyceride (Figure 3.13A) or total cholesterol (Figure 3.13B) in TRL, LDL and HDL plasma fractions compared to control mice.

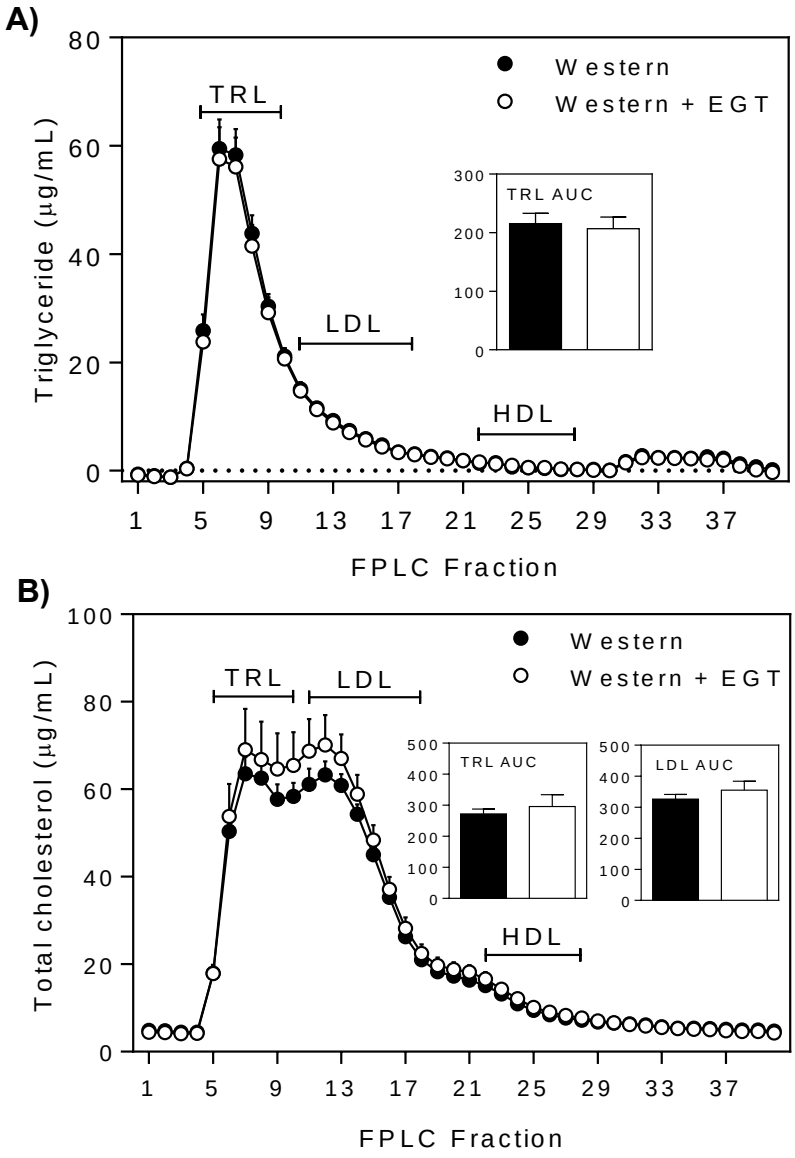

A)
Triglyceride (µg/mL)
80
60
40
20
0
TRL
LDL
HDL
Western
Western + EGT
TRL AUC
300
200
100
0
1
5
9
13
17
21
25
29
33
37
FPLC Fraction
B)
Total cholesterol (µg/mL)
100
80
60
40
20
0
TRL
LDL
HDL
Western
Western + EGT
TRL AUC
LDL AUC
500
400
300
200
100
0
1
5
9
13
17
21
25
29
33
37
FPLC Fraction

Figure 3.13 EGT supplementation does not affect lipoprotein profiles of diet-induced obese, hyperlipidemic mice

Six-week-old, male C57BL/6J LDLr$^{-/-}$ mice were fed western diet (42 % kcal from fat, 0.2 % kcal from cholesterol) *ad libitum* for a total of 16 weeks. On week 13, mice were put into individual cages and randomly assigned to two groups to continue western diet supplemented with or without EGT (40 mg/kg/day) for 3 weeks. At sacrifice, blood was collected and EDTA plasma was separated by fast protein liquid chromatography (FPLC). (A) Triglyceride and (B) total cholesterol concentrations were determined in triglyceride-rich lipoprotein (TRL), low density lipoprotein (LDL), and high-density lipoprotein (HDL) fractions using enzymatic, colorimetric assays. Areas under the curves (insets) were calculated for each lipoprotein class. Data are means ± SEM. n=7.

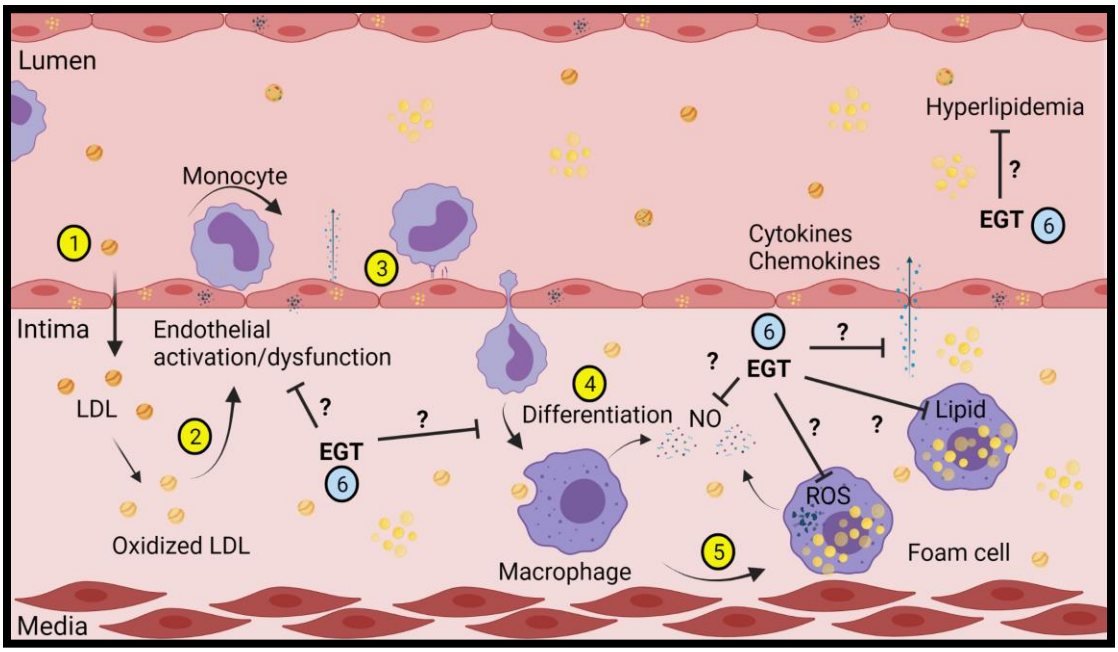
Chapter 4

4 Discussion

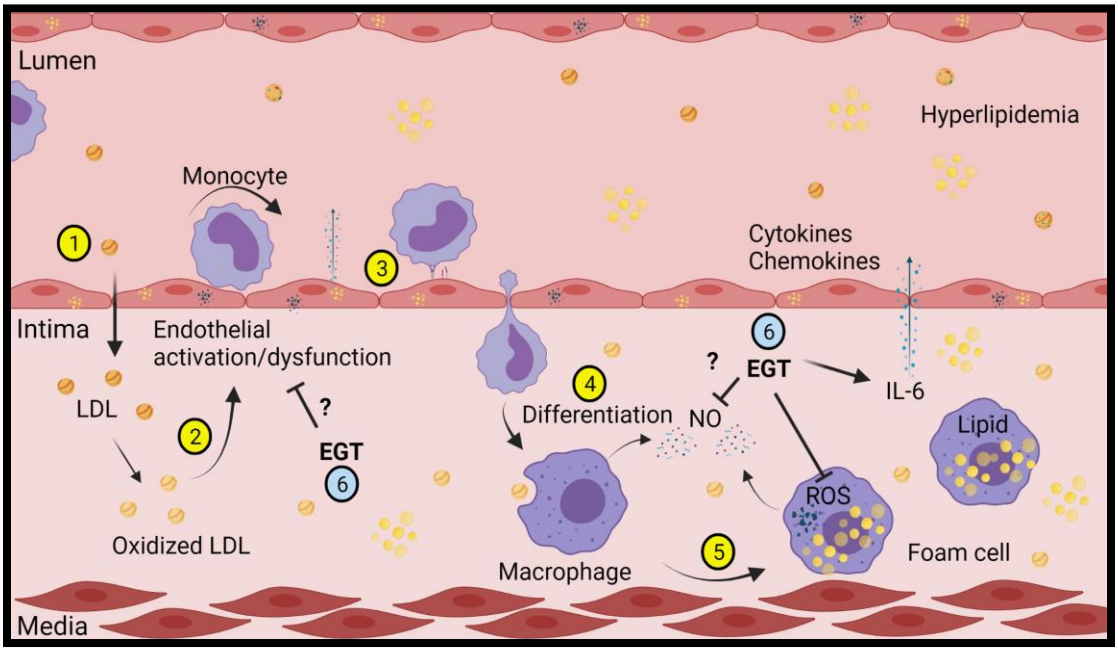
4.1 Summary of Results

Elevated concentrations of circulating lipids are observed in individuals who suffer from obesity and metabolic syndrome and significantly increase the risk of developing vascular disease [13]. Vascular endothelial cells and macrophages are vulnerable to chronic exposure to elevated plasma lipids, which induce oxidative stress and inflammation [25,27]. Consequently, endothelial dysfunction and pro-inflammatory activation of macrophages arises within the vasculature [25,27]. Figure 4.1A summarizes the potential targets of EGT that can modify vascular disease risk, which include endothelial cells, macrophages, and blood lipids [17]. EGT has been identified to have antioxidant and potential anti-inflammatory properties in cells exposed to various stressors [51,52,60]. In addition, EGT accumulates in high concentrations within many tissues that experience oxidative stress [52]. Some evidence has suggested that EGT could modify vascular disease risk by altering blood lipid profiles [64]. Currently, there are no published studies of the effects of EGT on vascular cells under high lipid conditions, or on blood lipid and lipoprotein profiles in diet-induced obese, hyperlipidemic mice. For this thesis, it was hypothesized that EGT supplementation would decrease endothelial cell dysfunction and macrophage inflammatory characteristics under high lipid conditions, and reduce markers of atherosclerotic risk in diet-induced obese, hyperlipidemic mice. The major findings of this thesis in relation to previously published studies are summarized in Figure 4.1 and include the following: **1)** EGT did not influence HMVEC tube formation or stability but demonstrated potential NO scavenging abilities by decreasing NO concentration in HMVEC; **2)** EGT showed no effect on monocyte to macrophage differentiation or on macrophage lipid accumulation under high PA/OA conditions; **3)** EGT induced IL-6 but not IL-1β secretion under high PA/OA conditions; **4)** EGT decreased cytosolic ROS and potentially NO accumulation in macrophages; **5)** EGT supplementation did not influence parameters of metabolic syndrome or lipoprotein profiles in diet-induced obese, hyperlipidemic mice.

A


Lumen
Hyperlipidemia
Monocyte
?
EGT
6
1
3
Cytokines
Chemokines
Intima
Endothelial
activation/dysfunction
6
?
?
EGT
LDL
4
Differentiation
NO
Lipid
2
?
?
?
EGT
6
?
?
ROS
Oxidized LDL
Macrophage
5
Foam cell
Media

B


Lumen
Hyperlipidemia
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Cytokines
Chemokines
Intima
Endothelial
activation/dysfunction
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EGT
IL-6
LDL
4
Differentiation
NO
Lipid
2
?
EGT
6
ROS
Oxidized LDL
Macrophage
5
Foam cell
Media

Figure 4.1 Summary of the effects of EGT on endothelial cell and macrophage function under high lipid conditions during vascular disease

(1A, B) During obesity and metabolic syndrome, elevated levels of circulating lipids induce endothelial cell damage which allows low density lipoprotein (LDL) particles to deposit into the intima. (2A, B) Once in the intima, LDL particles undergo oxidation, leading to the activation of endothelial cells. These activated endothelial cells start expressing adhesion molecules at their cell surface and release pro-inflammatory cytokines and chemokines into the blood circulation. (3A, B) In response, circulating monocytes are recruited to the vascular wall where they adhere to endothelial cells and migrate into the intima. (4A, B) Infiltrating monocytes differentiate into macrophages, phagocytose oxidized LDL and secrete a variety of pro-inflammatory cytokines, reactive oxygen species (ROS) and nitric oxide (NO) into the extracellular matrix. This results in further recruitment of macrophages and propagation of inflammation. (5A, B) During chronic exposure to lipids and lipoproteins such as LDL, macrophages become increasingly lipid-laden and are known as foam cells. Foam cell and lipid accumulation in the intima intensifies the inflammatory environment leading to the development and progression of atherosclerosis. (6A) Based on previously published studies, EGT was predicted to help protect against lipid induced oxidative stress and inflammation within the vasculature by decreasing NO and ROS accumulation, hyperlipidemia, and foam cell lipid accumulation. (6B) The studies presented in this thesis suggest that EGT decreases ROS, and potentially NO accumulation in vascular cells *in vitro* but has no effect on parameters of metabolic syndrome *in vivo*. Figure was created in BioRender.

4.2 EGT modulates aspects of endothelial cell function under high fatty acid conditions

Several *in vitro* studies have indicated that EGT may protect against glucose-induced endothelial dysfunction by modulating cellular oxidative stress, inflammatory and survival pathways [74,84,94]. The ability of EGT to reduce oxidative stress may be attributed to its ability to directly scavenge ROS, to downregulate the ROS producing enzyme NADPH oxidase 1, or to result in the induction of antioxidant enzymes, including glutathione reductase, catalase and superoxide dismutase [74]. However, the effects of EGT on endothelial cell function exposed to lipid induced oxidative stress are unknown. Therefore, this thesis investigated whether EGT would show similar beneficial effects on endothelial cell function under high lipid conditions rather than high-glucose conditions. To assess endothelial cell function, this thesis evaluated the effects of EGT on HMVEC tube formation and stability as well as NO concentration under high fatty acid conditions.

As described in section 3.2, EGT did not affect HMVEC tube formation or stability. It is possible that the design of these experiments did not allow sufficient time for EGT to accumulate within HMVEC before exposure to high fatty acid conditions. EGT cannot passively diffuse across the cell membrane, but rather is actively transported into the cell by OCTN1, which is a time dependent process. Previous studies have shown high accumulation of EGT in endothelial cells after 8 hours of incubation [84]. Moreover, EGT can regulate gene expression in endothelial cells to protect against oxidative stress and inflammation [91,94]. Therefore, follow up experiments were performed to determine whether pre-incubation of HMVEC with EGT for 8 hours prior to high fatty acid treatment to allow enough time for EGT to accumulate within HMVEC and to elicit any potential changes in gene expression would influence HMVEC tube formation and stability. Results again indicated that pre-incubation with EGT did not influence HMVEC tube formation and stability under high fatty acid conditions. The high fatty acid conditions used for these experiments, which included a mixture of palmitate plus oleate, did not significantly effect HMVEC tube formation or stability, which suggests that the unsaturated fatty acid, oleate, may be mitigating the toxic effects of the saturated fatty acid, palmitate [132]. Similar

observations have been made by our lab previously [124]. It is possible that a protective effect of EGT would be more easily observed under more toxic conditions, such as high palmitate in the absence of oleate. However, the combination of palmitate plus oleate is more representative of the fatty acid composition of western diet [121]. In follow up experiments, the additional cellular stress of hypoxia (2% oxygen) was included, which is observed in some vascular beds during ischemic vascular disease. Once again, there were no significant effects of EGT on HMVEC tube formation or stability. Based on these data, it was concluded that EGT does not significantly influence this aspect of endothelial cell function under high fatty acid conditions.

In contrast to tube formation, EGT did exert a significant effect on HMVEC NO concentration, which was significantly decreased under both basal and high fatty acid conditions. This suggests that EGT may be scavenging NO or affecting the expression or activation of NO regulators such as eNOS in HMVEC, which could either be detrimental or beneficial. On one hand, NO is important for endothelial function and decreased NO bioavailability is a known hallmark of endothelial dysfunction [35]. On the other hand, EGT can scavenge RNS such as peroxynitrite which can be formed when NO reacts with ROS induced by high lipid conditions [133,134]. This may suggest that EGT can either prevent the formation of peroxynitrite by decreasing NO concentration or scavenge peroxynitrite itself in the setting of lipid induced oxidative stress. Overall, our HMVEC *in vitro* studies suggest that EGT does not influence angiogenic function, as assessed by tube formation and stability, but it may have some beneficial or detrimental effects when decreasing NO concentration under high fatty acid conditions.

4.3 EGT modulates aspects of macrophage function under high fatty acid conditions

Macrophages, which differentiate from circulating monocytes, play a crucial role in the inflammatory response during chronic exposure to high vascular lipid deposition seen in individuals who suffer from obesity and metabolic syndrome [135]. The chronic inflammatory response in the vascular wall is sustained through secreted pro-inflammatory cytokines, chemokines, ROS and NO by macrophages and their lipid-laden counterparts,

foam cells. As a result, more monocytes are recruited to the vasculature, which leads to the progression of vascular disease [130].

EGT can decrease the binding between human monocytes and endothelial cells, a process which plays a critical part in recruiting monocytes into the vasculature [102]. For this thesis, multiple additional factors involving macrophages and that are known to influence the development of vascular disease under high lipid conditions were investigated, including monocyte to macrophage differentiation, macrophage NO, ROS and inflammatory cytokine production, and macrophage lipid accumulation (foam cell formation) as described in sections 3.5 to 3.9.

EGT did not affect monocyte to macrophage differentiation, macrophage NO concentration, or macrophage lipid accumulation. In the case of NO concentration, the discrepancy between the effect of EGT on macrophages compared to HMVEC may be due to the amount of NO produced by each cell type. The THP-1 macrophage cultures used in this study produced approximately 6 times more NO than HMVEC. This may suggest that the NO scavenging ability of EGT is dose dependent, such that the 0.25 mM concentration of EGT used in these experiments were able to decrease the relatively low concentrations of NO produced by HMVEC, however, could not significantly decrease the much higher concentrations produced by macrophages. Despite these observations, potentially protective effects of EGT may be targeting the downstream by-products of high fatty acid conditions rather than directly affecting lipid accumulation within macrophages.

In contrast to the lack of effects summarized above, a significant effect of EGT was observed on ROS accumulation and on IL-6 secretion under high fatty acid conditions. The finding of decreased ROS accumulation in macrophages exposed to high fatty acids in the presence of EGT are well supported by previous *in vitro* studies demonstrating the ROS scavenging capabilities of EGT in other cell types in response to other inducers of oxidative stress [66,84,92,136]. Recent studies have suggested that EGT may reduce the initial inflammatory response during vascular disease in endothelial cells. Koh et al. (2021) demonstrated that EGT can downregulate mRNA expression of pro-inflammatory cytokines IL-1β, IL-6, IL-8, TNF-α and cyclooxygenase-2 (COX2) in endothelial cells

treated with 7-ketocholeserol; a cholesterol oxidation product found in atherosclerosis [91]. In this thesis, macrophages exposed to high fatty acid conditions showed increased IL-1β but not IL-6 secretion. However, EGT increased IL-6 and not IL-1β secretion under high fatty acid conditions, suggesting that EGT may only modulate specific inflammatory cytokines, and could act as a pro-inflammatory modulator. This finding is consistent with studies performed by Yoshida et al. (2017), who demonstrated that EGT increased the secretion of the pro-inflammatory cytokines IL-6 and IL-12p40, and decreased the secretion of the anti-inflammatory cytokine IL-10 in toll-like receptor stimulated macrophages [137]. Taken together, these observations suggest that EGT may act as an anti- or pro-inflammatory modulator depending on the cell stress-inducers and cell types involved.

Overall, the *in vitro* work in this thesis suggests that EGT has moderate effects on vascular cells under high lipid conditions. EGT was shown to decrease HMVEC NO concentration and to increase IL-6 secretion and reduce cytosolic ROS accumulation in macrophages.

4.4 EGT does not influence characteristics of metabolic syndrome, as markers of vascular disease risk, in diet-induced obese, hyperlipidemic mice

A limited number of *in vivo* studies have investigated the effects of EGT in diet-induced obese animals. Some studies have investigated the potential effects of EGT on NAFLD which, as described in section 1.1 is associated with a cluster of metabolic abnormalities related to metabolic syndrome and significantly increases vascular disease risk [13]. In a high cholesterol-fed guinea pig model of NAFLD, EGT accumulated in liver tissue which was suggested to be a defensive mechanism initiated by this organ [80]. Moreover, mice fed an atherogenic high fat diet showed increased expression of hepatic OCTN1 in association with liver fibrosis, which was again suggested to be a defensive mechanism initiated by the liver [138]. However, these studies did not include EGT treatment groups, and their conclusions were solely based on associations. For this thesis, a dietary EGT supplementation intervention study was performed in male western diet-induced obese,

hyperlipidemic mice. At the conclusion of the study, risk factors for vascular disease including body weight, epididymal fat weight, liver triglyceride and cholesterol, plasma insulin, and blood glucose levels were assessed, and no significant differences were found between the control and EGT supplementation groups. In addition, plasma triglyceride, cholesterol, and lipoprotein profiles were assessed, as a previously published study suggested that EGT may decrease postprandial triglycerides and thereby modify vascular disease risk [64]. Again, no differences were observed between the control and EGT supplementation groups. Overall, the *in vivo* work in this thesis suggests that EGT does not influence parameters of metabolic syndrome and blood lipid levels which are risk factors for vascular disease in diet-induced obese, hyperlipidemic mice.

Several studies reviewed in a recent article by Tun et al. (2020) have suggested the potential usage of antioxidants as treatment for individuals who suffer from obesity and metabolic syndrome [139]. Many antioxidants including those from natural food sources such as vitamin E, polyphenols and carotenoids have been shown to decrease plasma and liver lipids, improve insulin sensitivity and reduce body weight [139]. However, other studies have found little to no effect. Hasty et al. (2007) showed no effects on systemic oxidative stress, hyperlipidemia, and obesity in obese, hyperlipidemic mice after 12 weeks of a vitamin E-enriched diet [140]. In addition, studies of supplementation with polyphenols such as resveratrol have shown no body weight or fat mass reduction in diet-induced obese animals [141–143]. The inconsistent findings between published antioxidant studies and the lack of statistically significant results in this thesis may be a result of dosage and treatment duration. For this thesis, the chosen EGT dose, 40 mg/kg/day, was based on a previous study by Tang et al. (2018) which showed significantly increased EGT accumulation in a variety of tissues, including the liver [79]. Although the dose of EGT provided was likely sufficient for EGT to accumulate in tissues, it may not accumulate to concentrations sufficient to alter metabolic pathways. Moreover, a longer dietary supplementation intervention with EGT may be required to observe any effects on the endpoints examined for this thesis.

4.5 Limitations and Future Directions

The *in vitro* studies described in this thesis have demonstrated potential antioxidant effects of EGT in vascular cells. THP-1 macrophages were shown to suppress ROS accumulation when treated with EGT under high lipid conditions. However, we did not determine whether these effects also occurred in endothelial cells. Previous studies have demonstrated that EGT decreases ROS accumulation in endothelial cells under high glucose conditions [74,84,94] suggesting that EGT may also protect endothelial cells against lipid induced oxidative stress. Furthermore, this thesis determined that EGT may have potential NO scavenging abilities in endothelial cells. Whether this is beneficial or detrimental to endothelial cells remains to be determined. Follow up studies should include measuring peroxynitrite concentrations to determine whether EGT helps prevent the formation of RNS. One limitation to this thesis work was the number of inflammatory cytokines measured. When assessing whether EGT influenced pro-inflammatory cytokines, increased secretion of IL-6 and not IL-1β was observed, which suggests that the effect of EGT may be cytokine selective. Future studies will need to investigate the effects on a broader range of pro-inflammatory cytokines such as TNF-α, IL-12 and IL-18 in addition to other anti-inflammatory cytokines such as IL-10, IL-19 and TGF-β to determine whether EGT acts as a pro- or anti-inflammatory modulator under high fatty acid conditions.

The *in vivo* studies described in this thesis clearly demonstrated a lack of effect of EGT dietary supplementation as an intervention to treat characteristics of metabolic syndrome that are markers of vascular disease risk. It would be of interest to determine the potential utility of EGT as a preventative treatment for obesity and vascular disease risk in future studies. It would also be of interest to determine whether EGT at a higher dose with a longer treatment period would influence characteristics of metabolic syndrome and atherosclerosis risk in diet-induced obese, hyperlipidemic mice. Moreover, follow up studies will determine whether studies conducted in endothelial cells and macrophages will be translatable to the *in vivo* model of diet-induced obesity and hyperlipidemia. In particular, future studies will determine whether EGT can protect against the development of atherosclerosis found within the aortic sinus of the mice used for this thesis work. Furthermore, other organs that were harvested at the time of sacrifice including the liver,

pancreas, ileum, jejunum, epididymal fat, and heart will also be examined to determine whether EGT has potentially beneficial effects in other metabolic tissues.

4.6 Conclusions

Vascular disease is a major complication of obesity and metabolic syndrome, which increases morbidity and mortality in individuals who suffer from these conditions. Lipid induced oxidative stress and inflammation that occur in vascular endothelial cells and macrophages are potential targets for the naturally derived antioxidant, EGT. This modified amino acid is increasingly recognized as an antioxidant and potential anti-inflammatory agent, which was explored further in vascular endothelial cells and macrophages under high lipid conditions in this thesis. It was determined that EGT had moderate effects on endothelial cell and macrophage function during exposure to high fatty acid conditions *in vitro*. Further studies in diet-induced obese, hyperlipidemic mice revealed no effects on characteristics of metabolic syndrome (blood glucose and blood lipids) as markers of atherosclerosis and vascular disease risk. Follow up studies based on results of the *in vitro* work in this thesis, in addition to examining aortic sinus tissue from the *in vivo* studies described here, will help determine the potential utility of EGT as a vascular health intervention supplement for individuals with obesity and metabolic syndrome.

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Appendices

Appendix A: Animal Use Protocol

AUP Number: 2020-073
PI Name: Borradaile, Nica M
AUP Title: Vascular disease in diet-induced metabolic syndrome
Approval Date: 10/01/2020

Official Notice of Animal Care Committee (ACC) Approval:
Your new Animal Use Protocol (AUP) 2020-073:1: entitled " Vascular disease in diet-induced metabolic syndrome"
has been APPROVED by the Animal Care Committee of the University Council on Animal Care. This approval, although valid for up to four years, is subject to annual Protocol Renewal.

Prior to commencing animal work, please review your AUP with your research team to ensure full understanding by everyone listed within this AUP.

As per your declaration within this approved AUP, you are obligated to ensure that:

1) Animals used in this research project will be cared for in alignment with:

a) Western's Senate MAPPs 7.12, 7.10, and 7.15
http://www.uwo.ca/univsec/policies_procedures/research.html

b) University Council on Animal Care Policies and related Animal Care Committee procedures

http://uwo.ca/research/services/animalethics/animal_care_and_use_policies.htm

2) As per UCAC's Animal Use Protocols Policy,

a) this AUP accurately represents intended animal use;
b) external approvals associated with this AUP, including permits and scientific/departmental peer approvals, are complete and accurate;
c) any divergence from this AUP will not be undertaken until the related Protocol Modification is approved by the ACC; and
d) AUP form submissions - Annual Protocol Renewals and Full AUP Renewals - will be submitted and attended to within timeframes outlined by the ACC.
e)
http://uwo.ca/research/services/animalethics/animal_use_protocols.html

3) As per MAPP 7.10 all individuals listed within this AUP as having any hands-on animal contact will

a) be made familiar with and have direct access to this AUP;

b) complete all required CCAC mandatory training (training@uwo.ca); and
c) be overseen by me to ensure appropriate care and use of animals.

4) As per MAPP 7.15,
a) Practice will align with approved AUP elements;
b) Unrestricted access to all animal areas will be given to ACVS Veterinarians and ACC Leaders;
c) UCAC policies and related ACC procedures will be followed, including but not limited to:
i) Research Animal Procurement
ii) Animal Care and Use Records
iii) Sick Animal Response
iv) Continuing Care Visits

5) As per institutional OH&S policies, all individuals listed within this AUP who will be using or potentially exposed to hazardous materials will have completed in advance the appropriate institutional OH&S training, facility-level training, and reviewed related (M)SDS Sheets,
http://www.uwo.ca/hr/learning/required/index.html

Submitted by: Copeman, Laura
on behalf of the Animal Care Committee
University Council on Animal Care

AUC Chair Signature

Dr.Timothy Regnault,
Animal Care Committee Chair

The University of Western Ontario
Animal Care Committee / University Council on Animal Care

Curriculum Vitae

Daniel Lam-Sidun

EDUCATION

September 2019-Present	MSc. Candidate, Physiology and Pharmacology Western University, London, ON, Canada
September 2015- April 2019	Bachelor of Medical Sciences (Western Scholars), Honours Specialization in Physiology & Pharmacology

ACADEMIC AWARDS

2020-2021	NSERC Canadian Graduate Scholarship – Masters

PUBLICATIONS

Daniel Lam-Sidun, Kia M. Peters, Nica M. Borradaile (2021). Mushroom-Derived Medicine? Preclinical Studies Suggest Potential Benefits of Ergothioneine for Cardiometabolic Health. *Int J Mol Sci.* 22(6): 3246.

Jake Pirkkanen, Sujeenthar Tharmalingam, Igor H. Morais, **Daniel Lam-Sidun,** Christopher Thome, Andrew M. Zarnke, Laura V. Benjamin, Adam C. Losch, Anthony J. Borgmann, Helen Chin Sinex, Marc S. Mendonca, Douglas R. Boreham (2019). Transcriptomic profiling of gamma ray induced mutants from the CGL1 human hybrid cell system reveals novel insights into the mechanisms of radiation-induced carcinogenesis. *Free Radic Biol Med*. 145: 300–311.

PRESENTATION & CONFERENCES

Lam-Sidun D, Peters K, Chen YJ, Chandrakumar R, Sawyez C, Borradaile N. The effects of ergothioneine on endothelial cell function and atherosclerosis in high lipid conditions. Physiology & Pharmacology Research Day, Virtual. November, 2020.

[Poster]

Lam-Sidun D, Peters K, Chen YJ, Chandrakumar R, Sawyez C, Borradaile N. The effect of ergothioneine on endothelial cell function and atherosclerosis under high lipid conditions. Canadian Lipoprotein Conference, Virtual, October, 2020.

[Oral Presentation]

Lam-Sidun D, Peters K, Chen YJ, Borradaile N. The effects of ergothioneine on endothelial cell function and atherosclerosis in high lipid conditions. Physiology & Pharmacology Research Day, Western University, London, Ontario. November, 2019.

[Poster]

TEACHING EXPERIENCE

September 2020-	Teaching Assistant, Western University, London, ON
April 2021	Department of Physiology and Pharmacology
	Pharmacology 3620 – *Human Pharmacology & Therapeutic Principles*
September 2019-	Teaching Assistant, Western University, London, ON
April 2020	Department of Physiology and Pharmacology
	Physiology and Pharmacology 3000E – *Laboratory Course*