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The Assessment of a Nano-Delivery System for Increasing the Bioavailability and Efficacy of Lutein in Preventing Hepatic Steatosis and Systemic Inflammation in Guinea Pigs

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University of Connecticut, 2016

Lutein is a xanthophyll synthesized by plants. In biological systems, lutein has antioxidant and anti-inflammatory properties, which could protect against chronic diseases. Despite its potential as a bioactive food component, lutein intake is low among Americans. In addition, lutein has low bioavailability, making it difficult to achieve biological effects. One approach to overcome these challenges is the use of nanotechnology.

This project evaluated the effects of a lutein nanoemulsion (NANO) on hepatic steatosis and systemic inflammation in guinea pigs. Our hypothesis was that NANO lutein would be more bioavailable and, therefore, more effective than powdered lutein (PL) in protecting against cholesterol-induced damage.

The nanoemulsion had an average particle size of 165.4 nm, a PDI of 0.195 and a ζ-potential of -40.5 mV, indicating a stable and monodisperse preparation.

Twenty-four male Hartley guinea pigs were allocated to one of three groups (n=8/group): control (0 mg lutein), PL (0.02% lutein) or NANO (0.02%

nanoemulsified lutein). All diets contained 0.25% cholesterol. After 6 wk, plasma, livers, and adipose tissue were collected for analysis. During the study, animals in both treatment groups consumed an average of 3.5 mg lutein/day. The NANO group had higher plasma and liver lutein concentrations compared to PL, indicating that lutein given in a nanoemulsion is more bioavailable.

In the liver, the NANO group had lower steatosis scores, accumulation of total and esterified cholesterol, and plasma ALT when compared to PL and control. Hepatic oxidized LDL was lower in both PL and NANO compared to control. Further, mRNA expression of DGAT2 was lower in the NANO group when compared to control or PL.

In plasma, the NANO group had higher LDL and HDL cholesterol concentrations and VLDL, LDL and HDL particle number compared to PL and control. Furthermore, the NANO group had 15% higher free cholesterol concentrations in adipose tissue, resulting in higher concentrations of inflammatory markers compared to the other treatments.

Overall, this nanoemulsion increased the bioavailability of lutein and protected the liver while plasma lipoproteins and systemic inflammation were increased, suggesting that the metabolic effects of this particular nanoemulsion are not protective to all tissues in guinea pigs.

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The Assessment of a Nano-Delivery System for Increasing the Bioavailability and Efficacy of Lutein in Preventing Hepatic Steatosis and Systemic Inflammation in Guinea Pigs

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Abbreviations

Acyl-CoA:cholesterol acyltransferase	ACAT
Age-related Macular Degeneration	AMD
Alanine Aminotransaminase	ALT
Ammonium	NH_4
Apolipoprotein A1	ApoA1
Apolipoprotein B	ApoB
Apolipoprotein E	ApoE
Aspartate Aminotransaminase	AST
ATP-binding Cassette	ABC
Bicinchoninic Acid	BCA
Butylated Hydroxytoluene	BHT
Cardiovascular Disease	CVD
Cholesterol Esters	CE
Cholesterol ester transfer protein	CETP
Chylomicron	CM
Cluster of Differentiation	CD
Complimentary Deoxyribonucleic Acid	cDNA
Deoxyribonucleic Acid	DNA
Diglyceride Acyl Transferase 2	DGAT2
D-α-tocopheryl polyeth+E18:I34ylene glycol 1000 succinate	TGPS
Enzyme-linked Immunosorbent Assay	ELISA
Esterified Cholesterol	EC
Food and Drug Administration	FDA
Free Cholesterol	FC
Free Fatty Acid	FFA
Hepatic Stellate Cells	HSCs
High Density Lipoprotein	HDL
High Density Lipoprotein Cholesterol	HDL-C
High Performance Liquid Chromatography	HPLC
Hydroxymethylglutaryl CoA reductase	HMGR
Insulin Resistance	IR
Interferon Gamma	IFNγ
Interleukin 10	IL-10
Interleukin 1-Beta	IL-1β
Interleukin 6	IL-6
Intermediate Density Lipoprotein	IDL
Lecithin-cholesterol acyl-transferase	LCAT
Lipoprotein Lipase	LPL

Liver X receptor alpha	LXRa
Low Density Lipoprotein	LDL
Low Density Lipoprotein Cholesterol	LDL-C
Low Density Lipoprotein Receptor	LDL-R
LDL-related protein 1	LRP1
Medium Chain Triglycerides	MCT
Messenger Ribonucleic Acid	mRNA
Methyl tert-butyl ether	MtBE
Metabolic syndrome	MetS
Mitogen-activated protein kinase	MAPK
Monocyte Chemoattractant Protein-1	MCP-1
Multi Drug Resistant Protein 1	MDR1
Nano Lutein Group	NANO
Niemann-Pick C1-like 1	NPC1L1
Non-Alcoholic Fatty Liver Disease	NAFLD
Non-alcoholic Steatohepatitis	NASH
Nuclear factor (erythroid-derived 2)-like 2	Nrf2
Nuclear Factor κB	NF-κB
Nuclear Magnetic Resonance	NMR
Oil in Water	O/W
Oxidized Low Density Lipoprotein	oxLDL
Polydispersion Index	PDI
Powdered Lutein	PL
Peroxisome proliferator-activated receptor gamma	PPARγ
Quantitative Real Time Polymerase Chain Reaction	qRT-PCR
Radioimmunoprecipitation assay buffer	RIPA
Reactive Oxygen Species	ROS
Reverse Cholesterol Transport	RCT
Scavenger Receptor	SR
Sodium Hydroxide	NaOH
Sterol Response Element-Binding Protein 2 (gene)	SREBPF2
Sterol Response Element-Binding Protein 2	SREBP2
Tocopheryl polyethylene glycol succinate	TPGS
Total Cholesterol	TC
Triglycerides	TG
Tumor Necrosis Factor-Alpha	TNFα
Type 2 Diabetes	T2DM
Very Low Density Lipoprotein	VLDL

Chapter 1

Introduction

One of the main concerns related to the western diet is the increased intake of certain nutrients such as saturated and trans fats, simple carbohydrates, and sodium, among others, and the negative effects that these have on human metabolism. In the United States, chronic diseases attributable to the western diet represent a serious threat to public health [1]. The increasing rates of obesity, type 2 diabetes (T2DM), atherosclerosis, non-alcoholic fatty liver disease (NAFLD), dyslipidemia, and cardiovascular disease (CVD) are strongly associated with non-healthy dietary patterns and low physical activity [2,3].

In contrast, epidemiological studies show a link between a high intake of plant-derived foods such as wine, fruits, nuts, vegetables, grains, legumes, and spices and a variety of health benefits such as lower incidence of metabolic syndrome (MetS), dyslipidemia, T2DM, CVD and certain types of cancer [4–7]. One of the reasons that consumption of these foods is associated with so many health benefits is the presence of non-nutrient secondary metabolites, also known as phytochemicals or bioactive components. These phytochemicals have been shown to exert a wide range of biological activities *in vitro* and *in vivo* [5]. Most phytochemicals have antioxidant capacity and since oxidative stress is thought to be involved in the etiology of many chronic diseases affecting the global population, these compounds are of great interest in health and nutrition research [8,9].

Among these phytochemicals, carotenoids, a family of pigmented and lipophilic compounds synthesized only by plants and some microorganisms, have received special attention [10–13]. For example, many studies have been conducted with lycopene and its relationship to prostate cancer [14], or with lutein/zeaxanthin and the prevention of age related macular degeneration (AMD) and the promotion of eye health [15–17]. Lutein is an oxygenated carotenoid that is particularly abundant in green leafy vegetables such as kale and spinach and yellow foods such as corn and egg yolks. In addition to being important for the maintenance of eye health, lutein may also enhance immune function, protect against cancer, CVD, and oxidative stress-related diseases [18–20].

Given the potential of bioactive components to prevent or modulate the high prevalence of chronic diseases, there is a large market for nutraceuticals which are defined as food-derived phytochemicals in pharmaceutical forms (pills, powders, capsules, vials etc.) [5]. Lutein is no exception, and this carotenoid can be found as an individual supplement or as a component of multivitamins, with the claim that its regular consumption can promote health [19].

However, as with other carotenoids and lipophilic phytochemicals, lutein has a very inconsistent and variable bioavailability [19,21,22]. This is because bioavailability of lutein depends on several factors including the food matrix, the source of lutein, the presence of other meal components such as fiber, fat or other carotenoids and the health status and genetics of the host [23–25]. Different approaches to enhance the absorption of lutein from foods or supplements are being studied [26,27]. One of these

approaches is nano-technology, which is the use of materials on the nanometer (10^{-9} m) scale designed to be transported in encapsulated carriers with improved water solubility, thermal stability, oral bioavailability and physiological performance [28]. This methodology has been successfully used with drugs, vaccines, and other vitamins and nutrients [29–32].

However, it is important to understand the differences in bioavailability, metabolism, tissue distribution, and even possible toxicity of these nanoparticle-delivered nutraceuticals [5]. For this reason, we developed a study that would characterize and evaluate the effects of feeding a nanoemulsion of lutein to cholesterol-challenged guinea pigs.

The main objective of this project is to develop and test a nanoemulsion of lutein in the protection of cholesterol-induced hepatic steatosis and systemic inflammation in guinea pigs. Our central hypothesis is that the nanoemulsion, by enhancing the bioavailability of lutein, will be more efficient than regular lutein in reversing the hepatic and systemic damage caused by the hypercholesterolemic diet in a guinea pig model.

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Chapter 2

Literature Review

2.1. Cholesterol

2.1.1 Cholesterol definition and cellular importance

Cholesterol is a non-energetic, 27 carbon, steroidal, unsaturated lipid that plays a vital role in cell biology [1,2]. It is an important component of cell membranes in higher eukaryotes, by regulating their fluidity, stability and permeability [3]. Cholesterol is also the precursor of vital biomolecules such as corticosteroids, bile acids, vitamin D and sexual hormones [1,4]. In mammals, most organs satisfy their needs for cholesterol via endogenous *de novo* biosynthesis from acetyl-CoA [1,5], however, 30% of the cholesterol pool is obtained from the diet (exogenous cholesterol), which many cells can internalize from lipoproteins [1,3,5].

In the cell, cholesterol can be found in two main forms: free or esterified with a ratio of approximately 3:7 [1]. The molecule is free when the hydroxyl group in carbon 3 can form hydrogen bonds or form an ester with a carboxylic acid, which means that the compound has some polarity; esterified is when the hydroxyl group has a fatty acid attached by an ester bond which makes the molecule highly hydrophobic [6].

2.1.1 Endogenous cholesterol synthesis

All nucleated cells can synthesize cholesterol through the mevalonate pathway. This process requires 20 enzymes to assemble 30 carbons from acetyl-Co A into a 27-carbon planar structure with four rings. The rate-limiting enzyme of this pathway is hydroxymethylglutaryl CoA reductase (HMGR), which catalyzes the synthesis of mevalonate from acetyl-CoA [3]. HMGR is tightly regulated through transcription, translation, post-translational modification and degradation [7]. This enzyme has been widely studied and it is the target for statins, a commonly used cholesterol lowering drug [8].

2.1.3 Dietary cholesterol absorption

In western societies, cholesterol intake is around 200–600 mg/day [9] from which 40-80% is absorbed [4]. Since this molecule is poorly soluble in water, it has to be emulsified upon intake with bile salts, cholesterol-derived amphiphilic molecules synthesized in the liver, which in contrast with their precursor, are very soluble given their capacity to self-associate [2,10]. This emulsification process is similar to that used for other fat components of the diet, including phospholipids and triglycerides [9]. About 10–15% of dietary cholesterol is esterified, and before absorption these esters are cleaved by a pancreatic enzyme, carboxyl ester lipase, however, most of the dietary cholesterol does not require pancreatic hydrolysis [9,11].

Although it is generally accepted that cholesterol is absorbed via passive diffusion by the enterocytes, studies have found that intestinal uptake of cholesterol occurs by protein-mediated intestinal uptake [9,10].

The main cholesterol transporter located in the apical membrane of enterocytes is identified as Niemann-Pick C1-like 1 (NPC1L1) protein [3,12,13]. Studies have also shown that there is another facilitated transport mechanism that sends intracellular sterols back into the small intestine for fecal excretion. These efflux transporters are the ATP-binding cassette (ABC) proteins ABCG5 and ABCG8 [12]. Once inside the intestinal cell, acyl-CoA:cholesterol acyltransferase (ACAT) catalyzes the esterfication of cholesterol; an important step prior to the packaging of cholesterol into nascent chylomicrons (CM) [3,4,12].

2.1.4 Cholesterol metabolism

As with other lipophilic compounds, cholesterol cannot be transported into the bloodstream without lipoproteins. These are amphipathic structures with a polar surface composed by a layer of phospholipids, free cholesterol and apo-proteins; and a non-polar core made up by triglycerides, cholesteryl esters (CE) and fat soluble vitamins [12]. When dietary cholesterol is transported from the intestine to inside the body, it is mainly carried by CM, a triglyceride rich lipoprotein characterized by the presence of ApoB-48 [14]. CM travel via the lymphatic system and reach the bloodstream at the level of the subclavian vein via the thoracic duct [15]. In the lymph and blood, CMs acquire other apo proteins such as apoCI, apoCII, apoCIII and apoE. ApoCII activates the endothelial cell-associated lipoprotein lipase (LPL), which hydrolyzes the triglycerides from the core of CMs and the resulting free fatty acids (FFA) are transported to the extra hepatic tissues for energetic needs or storage. LPL mediated

triglyceride hydrolysis decreases the size and increases the density of the particles, leaving CM ‘remnant’ particles, which are enriched in CE. CM remnants are taken up by the liver via the hepatic LDL receptor (LDL-R) and LDLR-related protein-1 (LRP1) through the interaction with ApoE. 12-14 hours after a meal, all CMs are cleared from circulation [12,16,17].

The liver synthesizes and secretes another class of triglyceride-rich lipoprotein, known as very low-density lipoprotein (VLDL). VLDL contains ApoB100, apoE, apoCII and apoCIII and is also packed up with endogenous cholesterol, lipid soluble vitamins and carotenoids [18]. VLDL is also a target for LPL, which hydrolyzes its triglyceride content, leaving a much denser, cholesterol-rich remnant particle and ultimately low-density lipoproteins (LDLs) [17,19].

High-density lipoproteins (HDL) play a crucial role in cholesterol metabolism, because they orchestrate reverse cholesterol transport (RCT), the transport of cholesterol from peripheral tissues to the liver [12]. An important feature of HDL is its maturation process. ApoA-I, expressed and secreted predominantly by the liver (70%) and small intestine (30%) acquires small amounts of phospholipids, creating very small HDL particles. These particles bind to ABCA1 in the liver, intestine, or macrophages, acquiring free cholesterol (FC) and increasing their lipid content, thus becoming discoid-shaped larger HDL particles or small HDL.

Lecithin-cholesterol acyl-transferase (LCAT) catalyzes the transfer of an acyl group from lecithin to FC and generates hydrophobic CE, which readily move to the core of HDL particles, producing a steady gradient of free cholesterol. This process promotes the efflux of cholesterol from cells like vascular macrophages and other peripheral tissues, thus leading to the formation of more spherical mature particles or medium HDL. Mature HDL bind with ABCG1 and scavenger receptor B1 (SR-BI), which are responsible for additional cholesterol efflux to mature HDL particles, further increasing the size and CE content [20–22].

Further, HDL remodeling by plasma and cell surface enzymes is mediated by ABCG1, hepatic lipase, endothelial lipase, cholesterol ester transfer protein (CETP), and phospholipid transfer protein. In humans, HDL-cholesterol (HDL-C) can be returned to the liver via two pathways: direct hepatic uptake by SR-B1; or through CETP exchange of CE for TG in apoB-containing lipoproteins (CM and VLDL), followed by hepatic uptake of these apoB-containing particles by the LDL receptor [23].

Multiple epidemiological studies have shown a strong association between high concentrations of HDL-C and reduced risk of CVD, and low concentrations of HDL-C with increased CVD. Therefore HDL-C is referred commonly as the “good” cholesterol [23]. However, besides promoting RCT, HDL particles also enhance antioxidant, anti-inflammatory, vasodilatory, and antithrombotic activities [24].

2.1.5 Cholesterol challenge

Since cholesterol plays an important role in normal metabolism, the cholesterol pool is tightly regulated, and either a major decrease or increase of this molecule is a risk factor for disease [1].

Numerous studies have suggested that dietary cholesterol increases plasma TC concentrations in humans and it has been shown that increases in LDL-C without a consequent increase in HDL-C is a major risk factor for atherosclerosis and CVD [25,26]. However, experimental data indicate that individuals vary considerably with respect to their response to dietary cholesterol and that dietary cholesterol does not change the LDL: HDL ratio [27]. Mean intakes in the U.S. are around 224 mg and 333 mg cholesterol per day, which should not represent an increased risk for CVD [26].

Cholesterol challenges, however, with dosages far above the usual intake, have been shown to alter homeostasis and develop metabolic disorders such as dyslipidemia, endothelial dysfunction, hepatic steatosis, CVD and systemic inflammation [1,28–30]. In guinea pigs, our laboratory has shown that a challenge of 0.25% of dietary cholesterol for six weeks results in hypercholesterolemia when compared to a control group (284 vs 48 mg/dL; p=0.0001) and elevated plasma activity of liver enzymes, Alanine amino transferase (ALT) and aspartate amino transferase (AST), which are biochemical indicators of hepatic damage. Moreover, the cholesterol challenge also caused hepatic steatosis, denoted by increased accumulation of TG along with total, free, and esterified cholesterol. In this time period, that amount of cholesterol was also capable of causing hepatic inflammation and injury seen as single cell necrosis in liver tissue [31].

2.2 Oxidative stress and Inflammation

2.2.1 Oxidative stress

A free radical is any chemical species that contains unpaired electrons. This unpaired electron usually produces a highly reactive free radical [32]. In biological systems, free radicals are formed as by-products of normal cellular function such as oxidative phosphorylation in the mitochondria [33,34]. These by-products are generally reactive oxygen species (ROS) as well as reactive nitrogen species [35].

Free radicals can cause damage to cellular proteins, membrane lipids and nucleic acids, and eventually cell death; however, the human body has several mechanisms to counteract this damage by the use of antioxidants, which are either naturally produced in situ (endogenous), or externally supplied through foods and/or supplements (exogenous) [35,36]. Based on their activity, antioxidants can be categorized as enzymatic when they break down and remove free radicals through enzymatic reactions. Examples of these antioxidants are superoxide dismutase, catalase and glutathione peroxidase. Non-enzymatic antioxidants work by interrupting free radical chain reactions, examples of the non-enzymatic antioxidants are vitamin C, vitamin E, polyphenols, carotenoids and glutathione [37].

When the production of pro-oxidant free radicals is higher than the organism's capacity to counteract their action with antioxidant systems, the organism faces oxidative stress [28,38,39]. It is hypothesized that oxidative stress plays a major role in the development and progression of chronic and degenerative diseases such as cancer, autoimmune

disorders, cataract, cardiovascular and neurodegenerative diseases, among others [35].

2.2.3 Chronic inflammation

Inflammation, under normal conditions, is an essential component of the innate immune response to pathogens and a protective mechanism against tissue damage [40]. However, the persistence of inflammation over a prolonged period increases the risk of cellular and tissue injury. This chronic inflammation is characterized by the up-regulation of systemic indicators such as leucocyte count, and serum and plasma concentrations of acute-phase proteins, pro-inflammatory cytokines, chemokines, soluble adhesion molecules and prothrombotic mediators [41].

Obesity, insulin resistance and T2DM are closely associated with low grade chronic inflammation, which is further considered the underlying mechanism for several metabolic complications such as MetS, atherosclerosis and certain types of cancer [42–44].

In obesity, the adipose tissue mediates many of these inflammatory responses [45]. The expansion of adipose tissue mass, the distinctive feature of obesity, causes a series of alterations which induce the secretion of a number of inflammatory mediators including Tumor Necrosis Factor-alpha (TNFα), interleukin (IL) -1β, IL-6, monocyte chemoattractant protein 1 (MCP-1). This production of inflammatory adipokines has been implicated in the development of insulin resistance and MetS [46].

Cholesterol metabolism also plays a role in inflammation. There is abundant evidence that the interaction of LDL with macrophages in atherosclerotic plaques leads to an increase in inflammatory gene expression [47]. Animal studies have shown that excess dietary cholesterol leads to an increase in macrophage accumulation in the adipose tissue, increasing insulin resistance and chronic systemic inflammation [48]. Accumulation of cholesterol in the adipocytes has been shown to be cytotoxic, inducing apoptosis and the recruitment of pro-inflammatory cells and aggravating the state of systemic inflammation [49,50]

2.2.4 Link between oxidative stress and inflammation

As previously stated, oxidative stress and inflammation are both linked to many chronic diseases, and a very important feature of these processes is that oxidative stress and inflammation are known to induce each other [40]. For example, ROS can stimulate the production of oxidized LDL (OxLDL), which is not recognized by the LDL receptor but can be taken up by macrophages. OxLDL induces the activation of peroxisome proliferator-activated receptor gamma (PPARγ), and influences the expression of downstream genes leading to foam cell formation, inflammation and atherosclerosis [51]. Some radicals like •O2- and H_2O_2 can also stimulate stress-related signaling mechanisms such as nuclear factor kB (NF-κB) and p38- (MAPK) leading to inflammatory responses [38]. Similarly, during inflammation, cells of the immune system such as macrophages and leucocytes are recruited to the site of damage. This results in a “respiratory burst,” an overproduction of ROS, and consequently oxidative stress [42].

Given the relationship of oxidative stress and inflammation, the use of antioxidants from natural sources has become increasingly important as therapeutic agents against oxidative stress and inflammation-related diseases [39,52–54]. While it is commonly believed that antioxidant bioactive components, given their capacity to scavenge ROS, have an indirect impact on inflammation, some authors suggest that these antioxidants are also capable of interacting directly with inflammatory pathways [42,55].

2.3 Non alcoholic fatty liver disease (NAFLD)

2.3.1 Definition

Nonalcoholic fatty liver disease (NAFLD) is the broad spectrum of hepatic diseases, which vary from nonalcoholic fatty liver (NAFL) to nonalcoholic steatohepatitis (NASH), which can progress to fibrosis, cirrhosis and hepatocellular carcinoma. The pathogenesis of NAFLD excludes excessive alcohol intake (less than 20 g of alcohol per day), use of steatogenic medication or hereditary disorders [56–59].

NAFL is defined as the presence of hepatic steatosis without ballooning degeneration and inflammation in the hepatocytes. This steatosis is product of the abnormal TG accumulation in hepatocytes as a result of an imbalance between the uptake of fat and its oxidation and/or export [58,60].

NASH is a necro-inflammatory disease, defined as the presence of diffuse fatty infiltration in the liver and characterized by ballooning and inflammation in the

hepatocytes with or without fibrosis. NAFL is mostly benign and risk of progression to cirrhosis and liver failure is minimal, whereas NASH can lead to further life threatening complications [56,59].

2.3.2 Epidemiology and importance

NAFLD is the most common cause of abnormal liver function among adults in the United States [61]. The prevalence of this disease ranges between 25-45% increasing in parallel with other chronic problems such as obesity. Current estimates suggest that 68% of US adults are overweight or obese; which means that 75 million and 100 million adult individuals in the United States likely have NAFLD [62]. The prevalence of NASH is less common, affecting an estimated 2–3% of the general population and up to 37% of the morbidly obese [63].

An alarming fact is that even though NAFLD is commonly referred as an exclusively adult disease, the rising prevalence of obesity and T2DM in children and adolescents over the last two decades has made NAFLD emerge as the leading cause of chronic liver disease in this segment of the population [62,64]. Moreover, it is predicted that by 2030, NALFD will be the most frequent indication for liver transplantation [65]. There is now growing evidence that NAFLD is not only confined to hepatic-related morbidity and mortality, but is a multisystem disease, affecting several extra-hepatic organs [65]

2.3.3 Pathogenesis

Insulin resistance (IR) is the most consistent underlying pathogenic factor for hepatic steatosis. IR increases lipolysis of peripheral fat through increased hormone-sensitive lipase activity with mobilization and uptake of free fatty acids by the liver [58,66]. However, most experts agree that fat accumulation in the hepatocytes is not enough to induce progressive liver damage, but it is the first step of a series of molecular and physiologic alterations that occur in the progression of NAFLD [64,66]. Triglyceride accumulation in hepatocytes increases the vulnerability of the liver to other insults through effects of cytokines or oxidative stress [67] since the circulating FFAs are not only substrate for TG synthesis but may also be directly cytotoxic [68].

Day *et al.* [69] proposed a "two-hit" hypothesis which states that after lipid accumulation in the liver (first hit) additional cofactors (hit 2) such as oxidative stress, lipotoxicity, adipocytokines, alterations in mitochondrial permeability and stellate cell activation, among others, are needed to induce persistent liver injury leading to NASH [69].

As stated previously, cholesterol feeding could induce dyslipidemia and insulin resistance. In animal models an excess of dietary cholesterol also appears to be an important risk factor for hepatic steatosis and progression to steatohepatitis. [29,67,70]

2.3.4 Dietary interventions

The increasing prevalence of NAFLD and comorbidities is likely caused by malnutrition and sedentary lifestyles characteristic of the modern world [60]. As unhealthy dietary patterns can lead to the development of this disease, several studies have

demonstrated a positive impact of dietary interventions in treating obesity, IR, and NAFLD [71].

Studies have shown a positive correlation between monounsaturated fatty acids and polyunsaturated fatty acids, specifically n-3, and decreased liver fat content, suggesting a protective role of these fatty acids in the progression of NAFLD [72]. In guinea pigs, a carbohydrate restriction diet showed to attenuate hepatic cholesterol accumulation induced by a cholesterol challenge [73]. These results have been also seen in humans, as low-carbohydrate diets has been shown to lead to lower serum ALT and hepatic steatosis [64].

Standard dietary interventions for NAFLD include macronutrient intake modifications such as carbohydrate and fat restriction; in addition, emerging dietary therapies for NAFLD include the use of antioxidants such as vitamins C, E and polyphenols, betaine, anthocyanins, curcumin and resveratrol [63,74]. Among these antioxidants, carotenoids have received special attention. In the National Health and Nutrition Examination Survey (NHANES) III, higher levels of serum carotenoids were associated with lower presence of apparent liver injury, as indicated by liver enzymes [75]

2.4 Carotenoids

2.4.1 Definition

Carotenoids are a class of isoprenoid compounds consisting of 40 carbon atoms known as tetraterpenoids [76]. These lipid soluble phytochemicals are mainly synthesized by plants and photosynthetic bacteria as pigments and they constitute part of the antioxidant machinery [77,78]. Carotenoids protect cellular structures against oxidative damage by scavenging singlet molecular oxygen and peroxyl radicals usually caused by light and normal plant metabolism [78–81]. There are more than 700 naturally-occurring carotenoids identified, but only around 50 are present in the human diet and among these, only 6 (beta-carotene, beta-cryptoxanthin, alpha-carotene, lycopene, lutein, and zeaxanthin) represent more than 95% of the carotenoids found in human blood [82].

Structurally, carotenoids can be classified into oxygen-lacking carotenes and oxygen-containing xanthophylls. The latter contain hydroxyl groups, which provide an amphipathic character, allowing xanthophylls to be inserted into the lipid bilayer of cell membranes and the outer monolayer of lipoproteins [83–85]. In recent years, special attention has been focused on biological activities of dietary xanthophylls such as lutein, zeaxanthin, β-cryptoxanthin, capsanthin, astaxanthin, and fucoxanthin [86].

Functionally, carotenoids can be categorized into pro and non pro vitamin A. Pro vitamin A carotenoids can be metabolized into retinal and retinol and therefore contribute to general growth, maintenance of visual function, regulation of differentiation of epithelial tissues, and embryonic development [87]. Examples of provitamin A carotenoids include beta-carotene, alpha-carotene and beta-cryptoxantin [88]. Conversely, non-pro vitamin

A carotenoids are those that do not yield vitamin A. Despite not having potential vitamin A function, non provitamin A carotenoids have been studied and associated with a variety of health beneficial effects [76,89]. This group includes lycopene, lutein, zeaxanthin and astaxanthin, among others [90,91].

Despite the fact that they are synthetized by animals and they are classified as organic compounds, carotenoids do not meet all the criteria for being considered vitamins and there is no daily recommended intake for them; however, lower serum concentrations of carotenoids have been positively associated with increased risk of CVD, T2DM, AMD, certain types of cancer and other chronic diseases [92–96]. Dietary sources of carotenoids include orange, red, and yellow fruits and green leafy vegetables, as well as egg yolk and some kinds of fish and crustaceans [82]. In most countries, however, 80–90% of human carotenoid intake comes from fruits and vegetables [82,97] .

2.4.2 Bioavailability

A very important aspect to consider when evaluating the effect of carotenoids in humans is the bioavailability of these compounds [86]. Bioavailability refers to the fraction of the ingested food component that is available for utilization or storage in normal physiological conditions [98]. It is futile to study in detail the health effects of dietary bioactive compounds if their bioavailability is not also fully understood [99]. For a bioactive to be bioavailable, it needs to be released from the food matrix after ingestion, be absorbed in the gastrointestinal tract, and be transported to the target tissue to exert a biological effect [99,100].

In the case of carotenoids, there are many factors that influence their bioavailability, which are commonly summarized by the mnemonic SLAMENGHI, grouped by De Pee & West in 1996 [101–103]. SLAMENGHI stands for

a. Species of carotenoids: This refers to the configuration of the carotenoid molecule. In plants, carotenoids are usually all-trans isomer. Cis isomers, however, can be formed during food processing and are more polar and more soluble in oil than trans isomers [101,104]. Probably the most relevant example of how this affects bioavailability is the case of lycopene, the most abundant carotenoid present in tomatoes [105]. When lycopene uptake from processed and unprocessed tomato juice was compared in humans, the cis isomer was better absorbed than the all-trans form [106].
b. Molecular Linkage: In plants, xanthophylls are often esterified with fatty acids [76,107]. For example, lutein can be found as a lutein palmitate diester or monoester in some fruits like peaches and oranges [84]. Carotenoid esters cannot be absorbed in the intestine, however, since no esterified xanthophylls are found in plasma after ingestion, it its thought that these can be de-esterified by brush border enzymes prior to absorption [76,104,108]. These enzymes are esterases and lipases that are stimulated by bile acids and therefore, as long as there is enough fat present in the meal, molecular linkage does not affect bioavailability to a great extent [84,109].
c. Amount of carotenoids consumed in a meal: Because all carotenoids follow the same absorptive pathway, these may interact with each other during intestinal

absorption and metabolism [84,101,108]. In a study that assessed whether carotenoids compete for intestinal absorption Tyssandier *et al*., reported that adding a second carotenoid to a meal that provided a first carotenoid diminished the chylomicron content of the first one suggesting that there may be a competition for absorption [110]. This competition can be at the level of micellar incorporation, intestinal uptake, lymphatic transport or at more than one level [76,103].

d. Matrix in which the carotenoid is incorporated: One of the main components of a compound's bioavailability is its bioaccessibility, which is the fraction of carotenoid ingested that is released to the gastrointestinal lumen and thereby available for absorption in the intestine [84,99]. For a carotenoid to be bioaccessible, it has to be released from its food matrix and be incorporated and dissolved into a hydrophobic domain (bulk lipid emulsion, or mixed micelles) before it can be potentially absorbed [103,104,111,112]. In general, carotenoids dissolved in oil are more bioavailable than carotenoids that come directly from their food source [82,101]. Within plants, carotenoids are typically found in chromoplasts and chloroplasts, where they are bound to complex proteins which would decrease their bioaccessibility [76,103]. Protein binding can explain the low bioavailability of carotenoids from dark-green leafy vegetables [101]. Besides, carotenoids are hardly released from raw vegetables due to the solid structure of the cell walls. In this case, processing including heat treatment can accelerate the release of carotenoids by destroying these structures [86,98].

e. Effectors of absorption and bioconversion: Many nutrients consumed together with carotenoids may affect carotenoid absorption, metabolism, transportation and/or bioconversion [101].

Dietary fat

In general, the presence of fat in the meal increases carotenoid solubilization, micellarization and secretion in chylomicrons and therefore, their bioavailability. Dietary fat also stimulates bile secretion and increases the quantity of carotenoids carried in micelles [76,103]. However, lipids do not influence all carotenoids to the same extent, being more important for highly lipophilic carotenes and esterified xanthophylls than for partially polar free xanthophylls [76,102]. Furthermore, the requirement for fat in the meal seems to be higher for unprocessed vegetables, than that for cooked meals [113].

Dietary Fiber

One of the main effects of dietary fiber on lipid metabolism relies on its interaction with bile acids, resulting in a lower lipase activity and decreased absorption of fats and fat-soluble substances, such as dietary cholesterol and carotenoids [76,101,111]. The presence of dietary fiber in fruits and vegetables may be another explanation for the lower bioavailability of carotenoids from plant-derived foods [103,112].

f. Nutrient and health status of the host: Some differences in absorption of carotenoids can be due to underlying nutritional problems with the host, for example, fat malabsorption can impair the uptake of lipid soluble nutrients, such as vitamins and carotenoids [84]. It has also been speculated that aging decreases the

absorption of nutrients because of age-related changes in gastrointestinal pH and digestive enzyme efficiency [76,114].

g. Genetic factors: In human studies, there is an important inter-individual variability in blood and tissue concentrations of carotenoids. Some individuals display little or no change in plasma concentration of carotenoids after supplementation, whereas others show important changes [108,115] In addition to the variable intake of these compounds, these differences can be explained by variants in genes encoding for the proteins involved in carotenoid absorption and metabolism [116]. This variation suggests that, as is the case with dietary cholesterol, there are hyper- and hypo-responders to carotenoids [25,115].

h. Host-related factors: Some of the differences observed in the serum response to ingestions of dietary carotenoids can be explained by differences in lifestyle and body composition of the host [101]. For example, a study by Gruber *et al.* found significant correlations between lower serum lutein and zeaxanthin and several factors such as smoking, heavy drinking, not being physically active, having higher percentage of fat mass, a higher waist-hip ratio, lower serum cholesterol, a higher white blood cell count, and high levels of C-reactive protein [117].

i. Interactions: This term refers to the differences that can be observed when two or more of the factors mentioned before play a role together and have a synergistic effect over carotenoids bioavailability compared to the effects observed by each factor independently [101].

Given the many factors that determine carotenoids' bioaccessibility, the amount of carotenoid bioavailable can vary from less than 10% to 50% of the amount consumed [118].

Improving bioactive components bioavailability has become an important focus in nutritional sciences research. With an increased bioavailability, carotenoids bioefficacy can also be improved [99,119]. Many approaches have been taken to overcome the low bioavailability obstacle of phytochemicals. These include chemical modification of the molecules of interest, design of colloidal systems to encapsulate them and the use of nanosystems [99].

2.4.3 Bioactivity

As mentioned before, epidemiological studies have suggested a positive link between higher consumption and tissue concentrations of carotenoids and lower risk of chronic disease [77,78]. The main mechanism by which carotenoids exert these beneficial effects is their antioxidant capacity [78,120,121].

Carotenoids belong to the most efficient physical quenchers of singlet oxygen [55], but these molecules also are able to scavange other free radicals of different origins, like peroxide radicals [80,81,55]. However, not all beneficial effects of carotenoids are related to their antioxidant activity [122], it is also hypothesized that carotenoids or their derivatives can interact with cysteine residues of the IKK and/or NF-κB subunits and inactivate the NF-κB pathway, thus decreasing inflammatory pathways directly [42]. Other mechanisms by which carotenoids may be beneficial for human health are their

impact on cellular signaling and their ability to modulate gene expression [42,80,81,55,122,123]. Another hypothesis on carotenoids action is the stimulatory effects exerted by carotenoids on gap junction communication, which is thought to be implicated in the regulation of cell growth, differentiation and apoptosis [122].

Furthermore, carotenoids and their metabolites have been shown to impact gene expression and cell function through multiple mechanisms, including the interaction/interference with several transcription factors and the modulation of signaling pathways associated with inflammatory and oxidative stress responses [124].

2.5 Lutein

2.5.1 Definition

The carotenoid that this project is focused on, is lutein. Lutein is a non-pro vitamin A carotenoid from the family of xanthophylls or oxycarotenoids, named on the bases of oxygen being part of its structure [84,86]. The biochemical structure of lutein and its stereoisomer zeaxanthin can be described as a long polyene chain with alternate conjugated double bonds and attached methyl side groups. At both ends of the carbon backbone, lutein contains a cyclic hexenyl structure with an attached hydroxyl group [85,125]. This particular structure provides xanthophylls with certain polarity relative to hydrocarbon carotenoids, which allows these molecules to be oriented perpendicular to the lipid membrane with the end hydroxyl groups exposed to an aqueous environment

[126,127]. The presence of these hydroxyl groups is also believed to play a critical role in the biologic function of lutein and zeaxanthin [84].

The structure of lutein also accounts for the absorbance of certain wavelengths of the visual light spectrum, with a peak of absorption at 446 nm, and the emission at other wavelengths leading to the characteristic yellow-orange color of this molecule [84,85]. The concentration of lutein is therefore responsible for the yellow coloration of certain animal tissues, such as the adipose tissue, the egg yolk and the macula lutea of primates [125,128].

2.5.2 Sources, intake and absorption

In foods, lutein is particularly abundant in green leafy vegetables such as kale (40 mg/100g) or spinach (12 mg/100g) and in yellow foods such as corn and egg yolks [84,129,130].

Because lutein has one hydroxyl group on each ionone ring, it can be esterified with fatty acids in plant cells, resulting in mono- and di-acylated derivatives [131]. The most common fatty acid that esterifies lutein is palmitic acid, giving origin to lutein palmitate, which is the most common form of lutein in vegetable sources [84]. Conversely, animal sources contain mostly free or unesterified lutein [85].

According to the United States Department of Agriculture (USDA), on average, the daily intake of lutein by Americans is about 1-2 mg per day [84], which is 60% lower than the

6mg per day that has been associated with protection against AMD [132]. This amount is not easy to achieve through diet, however, in some populations such as the South Pacific Islanders, the intake of lutein can be as high as 25 mg per day [133].

Once consumed, lutein has to be released from the food matrix and incorporated into mixed micelles which are molecular aggregates that transport fat-soluble nutrients, making them potentially accessible by the intestinal epithelium [112]. In humans, lutein is absorbed mainly in the small intestine, although in vitro studies have suggested that it is possible for lutein to also be absorbed in the colon [76]. The majority of the uptake of lutein by the enterocytes is thought to happen by simple diffusion. According to this mechanism, the micelles migrate through the unstirred water layer to the brush border membrane, where the micelles collide with the cellular membrane and lutein leaves the micellar structure and diffuses through the membrane into the cytoplasm of the enterocytes [86,102,112].

However, results from several studies have suggested that carotenoid absorption follows a dose dependent, saturable process, which cannot be explained by a simple diffusion mechanism alone. This indicates the involvement of facilitated transport in the uptake of lutein [76,85,102]. The participation of a transporter also could explain why, when eaten with other carotenoids, there is a competition for intestinal uptake [76].

Studies have reported the existence of receptor-mediated transport of lutein in the apical membrane of enterocytes, with strong indications for the involvement of the SR-

BI, a member of the ABC transporter super-family [102]. This transporter is located in various tissues including the liver, testis, ovaries, and macrophages but it can also be found in the brush border membrane of the enterocyte from the duodenum to the colon [134]. At the intestinal level, SR-BI facilitates the uptake of free and esterified cholesterol, phospholipids, and triacylglycerol hydrolysis products, indicating low substrate specificity [85,116]. In 2005, Reboul *et al.* identified SR-BI for the first time to play a role in the uptake of lutein at the intestinal level using Caco-2 cells [135].

Another protein thought to be involved in the intestinal uptake of lutein is the NPC1L1 [82]. This protein is widely expressed in human tissues, especially in the liver and the apical membrane of the enterocyte, where is the main cholesterol and phytosterol transporter [13]. A study done by During *et al.* [136] with Ezetimibe, a drug used to decrease dietary cholesterol absorption by inhibiting NPC1L1, showed that it significantly decreased the uptake of several carotenoids, including lutein in Caco-2 cells [136]. These results suggest that lutein and cholesterol share transporters for uptake in the intestine. Once inside the enterocyte, lutein has three possible fates.

A. **Incorporation into chylomicrons:** With most TG and dietary cholesterol, lutein is incorporated into CM and transported via the lymphatic system to the liver, where it can be stored or further packed into VLDL to be transported throughout the body [78,85,116].
B. **Uptake by HDL particles:** Unlike non-polar carotenes, that are mainly carried in LDL particles [130,137–139], xanthophylls including lutein and zeaxanthin are equally distributed between LDL and HDL particles [130,137–139]. Although lutein is thought to be absorbed exclusively via a chylomicron-mediated pathway and

transferred to HDL from VLDL via CETP, there is recent evidence that this carotenoid can also be incorporated into nascent HDL particles at the intestinal level [116,140]. *In vitro* studies with Caco-2 cells have shown that ABCA1 located at the basolateral membrane of enterocytes can transfer both lutein and zeaxanthin to apoA1, not to mature HDL [141]. These results are consistent with animal studies. For example, Niesso*r et al*. reported markedly increased plasma and liver lutein and zeaxanthin in hamsters treated with the LXR agonist T0901317, which is an ABCA1 inducer [141].

C. **Efflux:** Some percentage of the lutein absorbed may be effluxed back to the intestinal lumen through ABC transporters such as ABCG5 and ABCG8. These are well known mediators of the excretion of dietary phytosterols [142]. However, ABCG5 polymorphisms have also been suggested to be associated with the lutein bioavailability from eggs in human subjects [143]. ABCG5 may excrete lutein and other highly polar epoxy xanthophylls to intestinal lumen [86]. Another transporter that may contribute to the efflux of lutein back to the lumen is the Multi-drug resistance protein 1 (MDR1), also known as P-glycoprotein, which is an efflux pump for lipid-soluble compounds and drugs [86,144]. It is suggested that the affinity of substrates for MDR1 is related to their polarity, and therefore highly polar xanthophylls such as lutein may be excreted by this membrane transporter [86].

2.5.3 Lutein bioavailability

As mentioned before, the intake of lutein from natural sources of lutein is low [84]. This low intake represents an obstacle to observe the bioactive effects of lutein in humans;

however, it is not the only challenge. As with other carotenoids, the bioavailability of lutein varies widely among healthy subjects and depends on multiple factors, which means that serum concentrations are difficult to predict from dietary intake alone [82,85,101,145]. For example, although the total xanthophyll content in egg yolks is 1.2 mg/100 g, which is low compared to the content in fruits and vegetables, the bioavailability is around 3 times greater than that from a vegetable source because it provides a fatty matrix and the lutein in animal sources is not esterified [85,129,146].

2.5.4 Lutein biological effects

Lutein and zeaxanthin are selectively taken up and accumulated in the macula, a small area of the retina responsible for central vision and visual acuity, and are the only carotenoids present in this tissue [84,147]. These xanthophylls are thought to be carried to the retina through HDL, which as mentioned before, is the lipoprotein primarily associated with lutein and zeaxanthin [85,148]. This specific relationship between HDL, lutein and the retina was shown in a study carried out by Connor *et al.* [137] using Wisconsin hypo alpha mutant (WHAM) chickens. These animals have very low levels of HDL due to a mutation in the ABCA1 gene and when these were fed a high-lutein diet, lutein concentrations increased in all tissues except the retina, suggesting the pivotal role of HDL in the delivery of carotenoids to retinal tissue [137,148].

Once in the retina, zeaxanthin is mostly found in the foveal region, whereas lutein is more abundant in the perifoveal region [149,150]. Together, these pigments are referred to as the macular pigment, where they provide protection to the retinal tissue against

the oxidative blue light [84,85,150,151]. It is no surprise that traditionally lutein and zeaxanthin are linked to eye health, specifically to the prevention of AMD [84,125,150,152,153] and other eye diseases, such as cataracts, retinal ischemia, uveitis, retinitis pigmentosa and diabetic rethinopathy [85,154,155].

However, other potential benefits of lutein have been identified, since lutein may also protect against oxidative stress related diseases, such as atherosclerosis, diabetic microvascular complications and CVD [84,89,156,157]. Some authors have also found effects of lutein in the immune response and inflammation [85].

Moreover, lutein is also found accumulated in the human brain and the concentration of carotenoid in this tissue has been positively correlated with cognitive function [158].

Lutein biological effects are due to its capacity to absorb blue light, which the most phototoxic visible light to which the retina is routinely exposed [159]. This explains why lutein is directly involved in eye health. The other mechanism is quenching ROS, which is why lutein has been related to a decreased risk for diseases where oxidative damage plays a role [84,85]. Lutein is very efficient in quenching lipid peroxide radicals [131].

2.6 The use of nano technology in nutraceutical delivery

One approach to increase the absorption and bioavailability of lutein is the use of nanotechnology. Nanotechnology is the science that designs, synthesizes, characterizes and applies materials and devices on the nanometer scale (one-billionth of a meter) [160]. In the pharmaceutical and nutraceutical industry this technology is

applied in the formulation of carriers that protect bioactive agents, such as drugs, vaccines, genes and nutrients from degradation or inactivation [161,162].

One of the most important applications for encapsulation of phytochemicals is to enhance their bioavailability by changing their pharmacokinetics and biodistribution [163,164]. Reviews on the influence of particle size on the biological fate of hydrophobic bioactive compounds, such as carotenoids, indicate that their bioavailability increases when the size of the particles encapsulating them are within the 100–1000 nm range. An increase in oral bioavailability is desirable since it means that a lower amount is needed to get the same biological effect [165].

2.6.1 Nanoemulsions

One example of the application of nano technology in the encapsulation of lipid soluble compounds is the use of emulsion-based delivery systems. Oil-in-water (O/W) emulsions can be prepared by solubilizing the lipophilic bioactive components within the oil phase, and then homogenizing this with an aqueous phase containing a water-soluble emulsifier or surfactant [162].

Emulsions in general are non-equilibrium systems, therefore cannot be formed spontaneously and consequently, energy input is required [166]. The size of the droplets produced depends on the composition of the system and the homogenization method used [162]. The formation of macroemulsions (where the droplet size is a few

micrometers) is fairly easy to achieve, however, to form droplets in the nano scale (20–500nm) is difficult and requires a large amount of surfactant and/ or energy [167].

Emulsions are thermodynamically unstable systems and their stability is affected mainly by four processes: creaming, coalescence, flocculation and Ostwald ripening [168]. However, due to their small droplet size, nanoemulsions have long term stability to these processes because the brownian motion and consequently the diffusion rate are higher than the sedimentation (or creaming) rate [166,169,170].

As mentioned before, carotenoids' lipophilic nature and insolubility present challenges to their delivery and absorption [168]. Nanoemulsions are a promising technique to encapsulate carotenoids and overcome these challenges. This approach has been used with other lipidic nutraceuticals including delta-tocopherol, gamma-tocopherol, curcumin, lutein, lycopene, beta-carotene and astaxanthin, among others obtaining positive results [162,168,170–172].

2.6.2 Nanoemulsion characterization and evaluation parameters

There are many factors that need to be taken into account in order to assess the in vivo fate of nano-delivery systems, for example: particle size, surface area, particle number, aggregation/agglomeration state, charge, and surface coatings, among others, are all likely to influence the biological availability and effects of an administered nano particle [173].

Particle size

The size of the particle is what makes nanotechnology so useful, and it is also one of the most important characteristics to evaluate [165,174]. Controlling the nanoparticle dimensions is important because it influences its physicochemical properties, functional performance and potential biological fate [165]. The same molecule, having a different size shows differences in particle uptake, deposition, and clearance [174,175].

It is important to measure the particle size of a nanoemulsion droplets for scientific, technological, and regulation reasons. A variety of analytical techniques are available that are capable of providing information about the size of the particles in nanoemulsions, including static light scattering, dynamic light scattering, and electron microscopy [165].

Count rate: The count rate is the average number of photons detected per unit time in kilo-counts per second. This is useful for determining the sample quality, by monitoring its stability as a function of time [176].

Zeta potential: Zeta potential (ζ-potential) is a scientific term for electrokinetic potential in colloidal systems which has a major effect on the various properties of nano-drug or nano-nutrient delivery systems [177]. In other words, it is the electrostatic potential at the electrical double layer surrounding a nanoparticle in solution [175].

Measurement of ζ-potential is currently a very straight forward way to characterize the surface of charged colloids, its unit is usually minivolts (mV) and the results from this measurement can give relevant information on concentration, distribution, adsorption, ionization, exposure or shielding of charged moieties [177]. Nanoparticles with a ζ-potential between −10 and +10 mV are considered neutral, while nanoparticles with ζ-potentials of greater than +30 mV are considered strongly cationic and of less than −30 mV are considered strongly anionic [175].

Polydispersity index

Generally, nanoparticle samples contain a distribution of different sized particles. It is therefore important to characterize both the central tendency and the degree of particle-to-particle size variability present in the sample [175,178]. The polydispersity index, or PDI is a metric for size variability. The value of PDI ranging from 0 to 0.5 is said to be monodisperse and homogenous, whereas a PDI higher than 0.5 indicate nonhomogenity and polydispersity. Basically, the higher the value of PDI, the lower will be uniformity of the droplet size of the nanoemulsion. Most authors accept PDI values less than 0.3 as optimal [179,180].

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Chapter 3

Experimental Design

3.1 Background

The overall goal of this project was to evaluate the efficacy of a nanoemulsion of lutein in preventing cholesterol-induced hepatic steatosis and systemic inflammation using a guinea pig model. As mentioned before, lutein is a non-provitamin A oxycarotenoid known for its antioxidant properties, which can be used in the prevention of many chronic diseases where oxidative stress plays an important role [1]. It has been shown that high fat diets and an excess of cholesterol induce oxidative stress and inflammation in animal models [2–5].

The effects of lutein in cholesterol-challenged guinea pigs have already been studied in our laboratory. After feeding guinea pigs a hypercholesterolemic diet (0.25% cholesterol) with or without 0.01g/100 g lutein, Kim *et al.* found that despite the expected injury caused by the cholesterol challenge, the animals receiving lutein had 43% lower hepatic free cholesterol than the controls. Lutein fed guinea pigs also had lower expression of TNF-α and lower NF-κB DNA binding activity than the control group which indicates that lutein has anti-inflammatory properties and could be used as a dietary approach to prevent hepatic steatosis. Beneficial effects were observed also in the eyes, where lutein protected against cholesterol-induced oxidative stress and inflammation [6].

Furthermore, lutein also provided protection against atherosclerosis by significantly reducing aortic cholesterol and malondialdehyde concentrations, oxLDL in plasma and

the aorta, pro-inflammatory cytokines in plasma, and early signs of atherosclerotic lesions in the aorta of guinea pigs [7].

However, as with other lipophilic compounds, the absorption of lutein is dependent upon several factors, which makes it difficult to obtain a reliable prediction of its bioavailability from food sources and meals [8–10]. For this reason, several approaches to increase or obtain a consistent bioavailability of carotenoids like lutein have been studied [11].

One of these approaches is the use of nano technology and controlled delivery systems. The effectiveness of nano carriers as delivery systems is based on the encapsulation of phytochemicals to enhance their bioavailability by changing their pharmacokinetics and/or distribution patterns [12]. In general, smaller particle sizes have been associated with increased bioavailability due to a greater surface to volume ratio [13–15].

This was confirmed by Vishwanathan *et al*. [16] in two crossover human studies that compared a lutein supplement pill to two different doses of a lutein nanoemulsion added to orange juice (6mg/day in study 1 or 2 mg/day lutein in study 2). Each intervention was done for one week with a 2-week washout phase between treatments. The results showed that the mean serum lutein concentrations (n = 9) increased by 104% ($P < 0.001$) after the 6 mg supplement and 167% ($P < 0.001$) after the 6 mg nanoemulsion phase. In study 2, mean serum lutein concentrations (n = 11) increased by 37% ($P < 0.05$) with the 2 mg supplement and 75% ($P < 0.001$) with the 2mg nanoemulsion. The

use of a nanoemulsion resulted in 31% ($P < 0.05$) and 28% ($P < 0.05$) greater serum lutein concentrations compared to the supplement [16].

Based on these previous studies, we hypothesized that the use of a nanoemulsion would increase the bioavailability of lutein and also be more effective than powdered lutein in reducing the detrimental effects of a cholesterol challenge in guinea pigs.

3.2 Nanoemulsion preparation and characterization

In order to enhance the bioavailability of lutein, we used an emulsion-based delivery system. Oil-in-water (O/W) emulsions can be prepared by solubilizing lipophilic bioactive components within the oil phase, and then homogenizing this with an aqueous phase containing a water-soluble emulsifier [17].

For this case, we selected the use of d-alpha tocopheryl polyethylene glycol succinate (TPGS) as a surfactant. TPGS is a water-soluble derivative of vitamin E, which is formed by esterification of alpha tocopherol succinate with polyethylene glycol [18]. TPGS is approved by the Food and Drug Administration as a safe pharmaceutical adjuvant and has been widely used as a solubilizer, oral absorption/bioavailability enhancer, emulsifier, and stabilizer in novel drug delivery systems with positive results [19].

The nano-emulsion was prepared by Designs for Health (Suffield, CT) using the following formula:

8.4% TPGS (Antares Health Products, Jonesborough, TN)

11.2% medium chain triglyceride (MCT) oil (60:40 mix of caprylic acid:capric acids)

70.4% water

10.0% lutein powder at 5% (Maypro Industries, Westchester, NY)

TPGS was first melted at 65-70°C on a stir plate, followed by the addition of the MCT oil. Lutein powder was added next and stirred until thoroughly mixed. Lastly, water that had been previously heated to the same temperature was added. The mixture was stirred on the stir plate until uniform, and then mixed with a high-speed homogenizer at 12,000 rpm for 10 minutes.

The nanoemulsion was characterized for particle size, ζ-potential and PDI. Particle size and PDI were measured using dynamic light scattering with a Zetasizer Nano ZS (Malvern, Westborough, MA), and ζ-potential was determined by laser Doppler micro electrophoresis technology with the same instrument. Samples were diluted appropriately with water to meet the sensitivity requirement of the equipment.

Once characterized, the nanoemulsion was sent to Research Diets, Inc (New Brunswick, NJ) to be incorporated into the experimental diets required for the study.

3.3 Experimental diets

This project required the development of three different diets:

A) CONTROL: a pelleted non-purified diet that met all the nutritional requirements

for guinea pigs plus 0.25% of dietary cholesterol, which is sufficient to ensure liver damage in the proposed time frame of the experiment [20].

B) POWDERED LUTEIN (PL): a pelleted non-purified diet that matched to the control plus 5% powdered lutein, for a final concentration of 0.02% lutein by weight (Maypro Industries, Westchester NY).

C) NANO: a non-purified diet matched to control plus a nanoemulsion of lutein, previously prepared by Designs for Health (Suffield, CT), for a final concentration of 0.02% lutein.

For all diets, all ingredients were homogeneously mixed with water (5% by weight), dyed, and pelleted via cold extrusion process. After pelleting, diets were dried at 88-92°F for 2 days to remove excess water. The complete diet composition is presented in **Table 3.1**.

For the confirmation of the presence of lutein in food a quantitative analysis was performed in triplicate for the three diets. For each trial, the weight of a single pellet of food was recorded. The pellet was then placed in a glass vial and 5 mL of technical grade acetone was added. Each pellet was pulverized manually with a metal rod and then allowed to soak in the acetone for 1 hour over ice in a dark room. After this time, the samples were gently pipetted into eppendorf tubes and centrifuged for 2 min at 13,600 x g. The supernatant was then transferred to a clean glass vial, and the absorption spectra were measured using acetone as blank. Lambert-Beer's Law was

used to determine the concentration of lutein in the individual pellets, and from this the concentration of lutein in mg per g of feed was extrapolated [21].

3.4 Animal model

For this project, we used the guinea pig *(Cavia porcellus)* model challenged with 0.25% cholesterol, which is equivalent to 1800 mg/day in humans [20].

The guinea pig is a very good model to study cholesterol and lipoprotein metabolism [22]. One of the main reasons is that unlike other animal models, guinea pigs have a very similar cholesterol distribution in plasma when compared to humans, which means that they carry most of their cholesterol in LDL [22,23]. These animals also have hepatic cholesterol metabolism comparable to what is observed in humans [24]. Other studies have reported the deleterious effects of dietary cholesterol in guinea pigs as their LDL and hepatic cholesterol increase in a dose-dependent manner [25,26].

For this project, twenty-four male Hartley guinea pigs weighing between 200 to 300 grams were housed in pairs in a light cycle room. During the first week of acclimation, the animals received a regular chow diet and water ad libitum. After acclimation, the cages were randomly assigned to one of three groups: CONTROL, POWDERED LUTEIN (PL) or NANO for a total of 4 cages/8 guinea pigs per group. The experimental diets were introduced gradually (25%, 50%, 75% and 100%) by mixing them with the regular chow to avoid food aversions in the guinea pigs. After a week, once the animals were consuming 100% experimental diets, the 6-week trial started.

Food consumption was monitored three times a week, weighing the food left on the feeder and subtracting that amount from the amount given two days prior. Once a week, the guinea pigs were weighed to evaluate growth and overall health.

After 6 weeks, guinea pigs were fasted for 12 hours to prepare them for sacrifice. Each animal was anesthetized via inhalation of isoflurane (1-chloro-2,2,2-trifluoroethyl difluoromethyl ether) [27]. When no response was observed after an extremity pinch the guinea pigs were killed with the heart puncture technique [28] in order to collect a large volume of good quality blood.

The blood collected from the heart was centrifuged immediately (2000 x g for 20 mins) to separate the plasma. Aortas, livers, eyes, and epididymal adipose tissue were also collected and preserved at -80°C for further analysis.

All animal experiments were performed in accordance with U.S. Public Health Service/ U.S. Department of Agriculture guidelines. Experimental protocols were approved by the University of Connecticut Institutional Animal Care and Use Committee, protocol #A11-023.

2.5 Results and discussion

2.5.1 Characterization of the nanoemulsion

In this study, the nanoemulsion diluted 1000 times had an average size of 165.4 nm **(Fig 2.1)** and a PDI of 0.195. A PDI of 0 to 0.5 indicates a narrow distribution [29]. Our lutein nanoemulsion fell within this range, suggesting that the particle size distribution was uniform [30]. For nanoemulsions, the desired PDI is < 0.3, as higher values have shown to increase Ostwald ripening and decrease stability [13,31].

ζ-potential is another important parameter of nano preparations. This value is an useful indicator of particle surface charge, which can be used to predict and control the stability, performance, and pharmacokinetic properties of colloidal suspensions or emulsions [14,32,33]. The nanoemulsion used in this study had a ζ-potential of -40.5 mV, which may be explained by the potent emulsification effect of TPGS, which solubilized MCT, resulting in the exposure of carboxyl groups on the fatty acids in the aqueous phase.

It is currently accepted that ζ-potentials under |30|mV are optimum, while potentials between |5| and |15|mV are in the region of limited flocculation; and between |5| and |3|mV of maximum flocculation. Thus, high values of ζ-potentials prevent particle aggregation and flocculation of a colloidal dispersion [33]. Our data indicates that the prepared lutein nanoemulsion consisted of well-distributed oil droplets with excellent storage stability.

2.5.2 Food intake

During the intervention, the guinea pigs consumed an average of 17 grams of food per day, which is equivalent to 3.5 mg of lutein/day for both PL and NANO groups. There was no difference in food intake (pooled mean ± SE: 17 ± 4 g/d) or final body weight among groups during the intervention (pooled mean ± SE: 528 ± 48 g) (**Table 3.2**).

2.5.3 Liver and adipose weight

There was no difference in weight of epididymal adipose tissue between groups. However, livers of the NANO group weighted less than the ones from other groups. Higher liver weight has been suggested to be correlated with increased hepatic TG, diglycerides, CE, and FC concentrations [2,34] Data are shown in **Table 3.2.**

2.6 Strengths and limitations

Strengths of this study design include the use of an animal model that mimics cholesterol metabolism in humans [22–24]. The guinea pig's size also facilitates the sacrifice process and tissue collection. This model has been used numerous times to evaluate the effects of low carbohydrate diets [20,35,36], fiber [37], cholesterol [35], dietary fat [26,38] lutein [6,7] and polyphenols [39], among others on various measures of health.

The main limitation of the study design was the lack of an empty vehicle control group. This addition would allow for an account of the effects of the components of the nanoemulsion without the lutein. In this particular nano formulation, there are two main components that could have influenced the results presented in this work: TPGS and MCT oil.

As mentioned previously, TPGS is esterified α-tocopherol succinate with polyethylene glycol 1000. TPGS, unlike free tocopherols, is water soluble and has been used to enhance the bioavailability and solubility of drugs and lipophilic compounds due to its capacity to form micelles and be absorbed without the need of bile salts [40]. However, TPGS can be also used as a vitamin E supplement [41,42] because the compound can release free tocopherol in the intestinal cell that can be absorbed and transported in lipoproteins like regular vitamin E [43]. Some authors have also reported that vitamin E can have synergistic effects with carotenoids, enhancing their antioxidant capacity [44].

Due to their lipophilic characteristics, intestinal absorption of carotenoids is enhanced by the presence of dietary lipids in the meal [45,46]. In this case, the absorption of lutein may have been facilitated by the presence of MCT in the nanoemulsion. Dietary fat facilitates solubilization, micellarization, and packaging of dietary carotenoids into chylomicrons [10,47]. In addition to the amount of fat, the structural characteristics of the dietary lipids also influence carotenoid bioavailability. Fatty acid chain length and degrees of saturation differ in their ability to enhance carotenoid absorption [47]. MCT contains fatty acids of 6 to 12 carbons and differ from long chain triglycerides in that they are absorbed directly into the portal circulation and transported to the liver without needing chylomicrons [48]. Some authors suggest that the presence of MCT could not only increase the bioavailability of carotenoids, but also affect their tissue distribution [47].

TABLES

Table 3.1 Composition of the experimental diets

Component	Control		PL		NANO	
	g/100 g	% Energy	g/100 g	% Energy	g/100 g	% Energy
Protein[1]	22.2	23	21.3	23	22.1	23
Carbohydrate	40.5	41.9	38.8	41.9	40.4	41.9
Fat[2]	15.1	35.1	14.4	35.1	15	35.1
Vitamins[3]	1.1	-	1.1	-	1.0	-
Minerals[3]	8.1	-	8.1	-	7.8	-
Cellulose	10.8	-	10.8	-	10.4	-
Guar Gum	2.7	-	2.7	-	2.6	-
Cholesterol	0.25	-	0.25	-	0.25	-
Lutein						
Powder [4]	0	-	0.021*	-	-	-
Nano-Lutein[5]	0	-	0	-	0.021*	-

[1] Soybean protein + 0.5 g/100g L-Methionine

[2] Fat mix was olive oil-palm kernel oil-safflower oil, high in lauric and myristic acids as previously reported [49]

[3]Compostion of vitamin and mineral mixes have been previously reported [50]

[4] Powdered lutein provided by Maypro Industries

[5] Nano-emulsion prepared by Designs for Health, Suffield, CT

*0.021% is equivalent to an intake of 3.5 mg of lutein per day based on food consumption

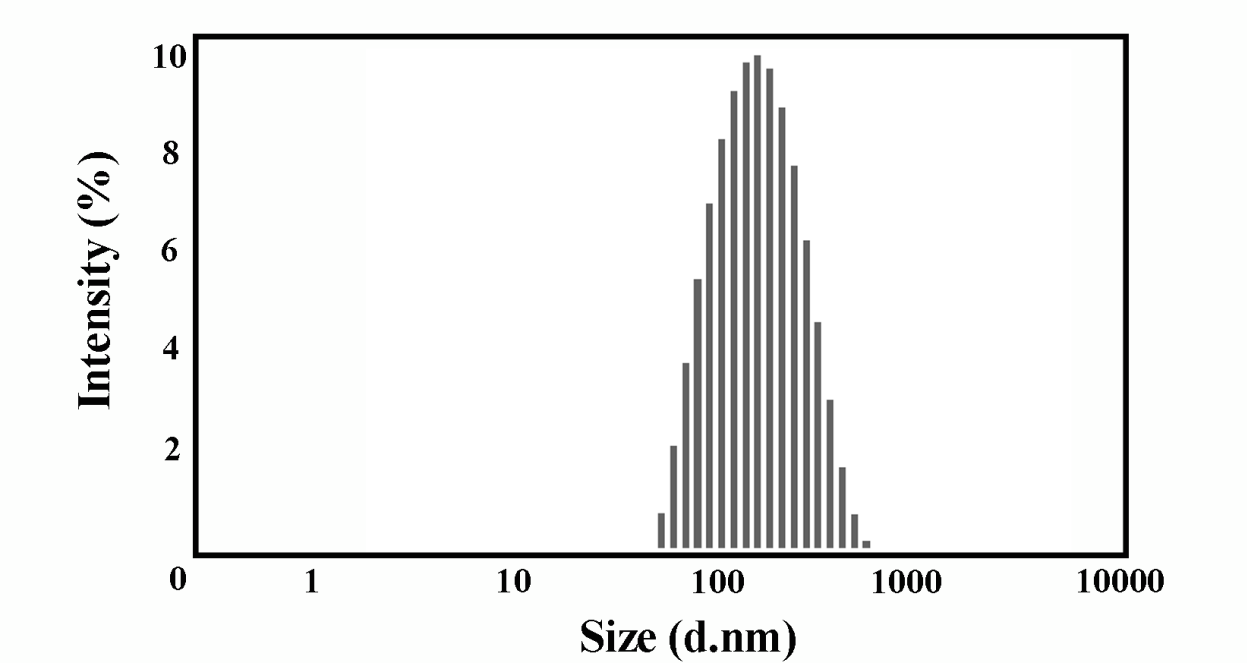
Table 3.2 Food intake, body, liver and adipose tissue weights of guinea pigs fed a hypercholesterolemic diet with no lutein (control) powdered lutein (PL) or nano-lutein (NANO)[1]

	Control	PL	NANO
Food intake (g/d)	16.7±0.9	18±1.0	16.0±1.3
Body Weight (g)	534.8±44	543±54	507±43
Liver weight (g)	24.6±2.8[a]	26.3±3.9[a]	21.5±2.9[b]
Adipose weight (g)	2.8±1.2	3.8±1.1	5.6±7.1

[1] Values are means ± SD, n=8. Values in the same row with different superscripts are significantly different at P< 0.05.

FIGURES

Figure 3.1 Average particle size distribution of the lutein nanoemulsion with a 1000X dilution



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Chapter 4

Evaluation of bioavailability of a nanoemulsion of lutein

4.1 Background

Many compounds found naturally in foods are thought to have beneficial effects on human health. However, these potential benefits, usually studied in vitro, are not observed in vivo because these compounds have relatively low and/or variable oral bioavailability [1–3]. Upon oral consumption, a certain percentage of micronutrients and phytochemicals are not absorbed. Bioavailability is defined as the fraction of an ingested nutrient that is available for utilization in normal physiological functions or for storage [4,5]. It is a complex concept, because it entails many processes from ingestion to delivery to the target organ or tissue while there is a wide variety of affecting factors [5]. Bioavailability can be further classified into four different steps which are bioaccessibility, absorption, tissue distribution, and bioactivity [6]. Bioaccessibility is the amount of ingested nutrient that is available for absorption in the intestine. This is the first challenge on improving bioavailability of nutrients, specifically carotenoids, because often the inefficient release from the food matrix results in poor absorption, and limited biological activity [7,8].

Bioavailability is usually measured by the amount of nutraceutical that is present in serum or plasma after ingestion. However, in the case of carotenoids, the concentration in plasma represents about 1% of the total body carotenoid content, whereas the majority of carotenoids are found in the liver [4]. This is why measurement of the concentration in target organs rather than plasma is a better way to understand the bioavailability of these compounds.

Multiple approaches have been taken to improve the bioavailability of nutraceuticals and increase the potential health benefits of these compounds without increasing the dosages. One of these approaches is the use of nanotechnology to encapsulate a poorly-absorbed compound to preserve its native properties over time and enhance its absorption [9]. Liposomes, micelles, nanoemulsions and polymeric nanoparticles have been tested for efficacy and safety, demonstrating a broad variety of useful properties [10]. Nanoemulsions are a class of small droplets usually in the range from 50 to 200nm [11]. The physicochemical characteristics (e.g., composition, dimensions, structure, charge, and physical state) of these nanoemulsions can be controlled by selection of appropriate ingredients and fabrication techniques, depending on the compound encapsulated and the desired effect of the preparation [12].

Nanoemulsions of lutein have been studied before with positive results [13]. In this project, the nanoemulsion described in chapter 3 was tested in terms of efficacy for increasing the bioavailability of lutein. Our hypothesis was that the nanoemulsion would improve the bioavailability of lutein, measured as lutein concentration in plasma and three target tissues: eyes, liver, and adipose.

4.2 Methods

4.2.1 Lutein extraction from plasma and tissues:

To assess the bioavailability of lutein in the three experimental groups mentioned in chapter 3, carotenoids were extracted from plasma, liver, adipose tissue and eyes and measured by high performance liquid chromatography (HPLC).

For plasma, the method followed is described by Blesso *et al.* [14]. Briefly, 200 µl of plasma was added into a 16x100 mm screw cap glass vial, followed by the addition of 150 µL of internal standard (trans-b-apo-8'-carotenal, Sigma Aldrich) diluted in ethanol, 500 µL of saline solution, and 2mL of a mixture of chloroform:methanol (2:1 v/v). The vials were then vortexed for 30 seconds and centrifuged at 2000 rpm for 10 mins at 4°C. After centrifugation, the chloroform layer was transferred to another vial using a Pasteur pipette. To the first vial, 3 mL of HPLC grade hexane was added to extract any remaining carotenoids. The vials were vortexed for 40 seconds and centrifuged at 2000 rpm for 10 min at 4°C. The organic phase was transferred to the vial containing methanol and dried under nitrogen gas in a 40°C water bath. The dried carotenoids were reconstituted in 150µL absolute ethanol and sonicated for 30 seconds. The reconstituted samples were transferred from the glass tube into a 1.5 mL amber microcentrifuge tube and spun at 14000 rpm for 10 min at 4°C. Finally, 150 µL of each solution was transferred to an amber HPLC vial.

For tissue samples, methods were adapted from Schmitz *et al.* [15]. Approximately 1 g of adipose tissue and liver or one eye were transferred to a 50-mL centrifuge tube containing 5 mL of an ethanol:water mixture (50:50 v/v) with 1% butylated hydroxytoluene (BHT) added as an antioxidant. Tissues were then homogenized completely with a tissue homogenizer followed by the addition of 5 mL 10% sodium

hydroxide (NaOH) in ethanol to the resulting slurry. The samples were mixed and saponified in a 60°C water bath for 30 min. All samples were then cooled in an ice bath and 10 mL of distilled water was added. 200 µl of this mixture was transferred to a glass vial and 150 µl of the internal standard was added. Each sample was extracted three times with 10 mL of hexane, with a centrifugation at 2000 rpm for 10 mins at 4°C after each extraction. Hexane was evaporated with nitrogen gas in a 40°C water bath and carotenoids were reconstituted with ethanol and transferred to an amber HPLC vial as detailed above. Analysis of plasma and tissue extract was completed within 48 h of the extraction.

4.2.2 HPLC analysis

Lutein analysis was conducted using a Dionex UltiMate 3000 UHPLC with a YMC Carotenoid 3 µm, 4.6 x 150mm column with a guard column. The autosampler was maintained at 5°C and the column oven at 25°C. The flow rate was 1 mL per minute for a total run time of 55 minutes. Mobile phases were (A) Methanol: Methyl tert-butyl ether (MtBE):ammonium (NH_4) acetate in water (83:15:2) and (B) Methanol:MtBE:NH_4 acetate in water (8:90:2). Mobile phase flow followed a gradient from 0-100% B over the 55 minute run time.

4.2.2 Statistical analysis

Differences between groups were analyzed by One-Way ANOVA with Fisher's LSD post hoc analysis. $P < 0.05$ was considered to be significant. Data are presented as mean ± SD. All analyses were conducted on SPSS for Windows, Version 20 (IBM

Corp.)

4.3 Results

The NANO group had a significantly higher concentration of lutein in both plasma (**Fig 4.1**) and liver (**Fig 4.2**) compared to the other treatments ($P < 0.001$). In contrast, although a trend is observed, there were no differences in lutein concentration between the lutein and NANO groups in the eyes (**Fig 4.3**) or the adipose tissue (**Fig 4.4**). However, carotenoid concentration in both PL and NANO were higher than the control group in these tissues ($P < 0.05$).

4.4 Discussion

In this study, we have demonstrated that a nanoemulsion of lutein is more bioavailable than regular powdered lutein in guinea pigs, as high concentrations of this carotenoid were found in liver and plasma from the NANO group. Similar results have been reported in humans, where a nanoemulsion of lutein was found to be associated with higher concentrations of plasma lutein [13]. Similarly, nanoemulsions have been used effectively with other lipid soluble compounds, such as β-carotene [16] and vitamin E [17], denoting the efficacy of this technology in increasing the absorption of poorly bioavailable compounds.

In this particular case, the use of TPGS as a surfactant could have also contributed to the increased bioavailability of lutein via inhibition of MDR1. As previously described, MDR1 is major efflux pump for lipid soluble compounds located, among other sites, in

the apical membrane of enterocytes, and it has been proposed that once absorbed, xanthophylls such as lutein may be effluxed back into the intestinal lumen via this transporter [18]. TPGS has shown to be an efficient MDR1 inhibitor in studies with several drugs, which resulted in an increased bioavailability of the compounds tested [19]. This is one of the reasons why TPGS has been extensively used in drug delivery studies and probably on of the reasons why, in the present study, there was more lutein in liver, plasma, eyes and adipose tissue of the guinea pigs of the NANO group when compared to controls and in plasma and liver when compared to PL.

Another possible explanation for the increased bioavailability of lutein is that nanoemulsion droplets have a higher surface area of the lipid phase exposed to the surrounding aqueous phase, which increases their lipid digestion rate [20,21]. This can be translated into more lutein available for absorption at the apical membrane of the intestinal cells. Moreover, studies have shown that, particularly for lutein, a slow transit time in the gastrointestinal tract is correlated with higher absorption [22]. One of the characteristics of nanoparticles is that their small size allows them to penetrate the intestinal mucus layer and become trapped (mucoadhesion), allowing more time for lutein, in this case, to be released and absorbed by the enterocyte [12,20].

An interesting observation is that lutein is selectively accumulated in eye tissues. However, in this particular study, although a trend is clear, there is no significant difference between the concentration of lutein in the eyes of the guinea pigs from the NANO group and the PL group. However, it is important to clarify that lutein and its

stereoisomer zeaxanthin are concentrated in the macular region of human and nonhuman primates, while guinea pigs, as with many other rodents, do not have a macula and lack major retinal vessels [23,24]. Therefore, it is unsurprising that the relationship between plasma lutein concentrations and eye lutein concentrations are not congruent. Nevertheless, lutein can also be found in small concentrations in the eye lens, which is why, despite the absence of a macula, lutein was found in both PL and NANO groups [25].

As mentioned previously, most carotenoids in the body are stored in the liver [4], where we found a higher concentration of lutein in the NANO group compared to the two other groups. This could explain why there was a lower concentration of this carotenoid in the adipose tissue when compared to the liver **(Fig 4.2 and Fig 4.4).** Furthermore, other researchers have found that lutein is preferentially accumulated in the liver, with less distribution in the adipose tissue, which is in agreement with our results [26,27].

4.5 Strengths and limitations

The main strength of this part of the study is the use of different tissues to measure bioavailability. This gives detailed information about the biological fate of lutein. Knowing that this carotenoid is preferentially accumulated in the eye in humans opened not only an entire area for research, but also a market for supplementation for the prevention of AMD and other eye diseases [25,28,29]. Since lutein is a potent antioxidant, it is possible that an alternate use of lutein supplementation would be for the prevention of liver diseases in which oxidative stress plays a major role.

One limitation of this study is the lack of other tissues of interest, such as spleen or brain, to evaluate lutein distribution. The most important tissue to collect would have been the intestine, in which we could have measured MDR1 expression and activity.

One reason why some tissue results were not significant, despite the evident trend, is the variability within the groups. This can be explained by the fact that the animals were eating *ad libitum* and they were caged in pairs, so the amount of lutein consumed by each individual animal may differ significantly, especially between paired guinea pigs, because there is usually dominance of one animal over the other. For feeding studies, oral gavage is usually the best choice, however, the guinea pig is not a good model to apply this technique as it tends to be painful and usually causes digestive tract injury or death by airway perfusion [30]. Also, more animals in each group could have added power to the statistics to reach significance in the trends.

Another limitation is the fact that the nanoemulsion added fat to the guinea pigs' diet. This addition may be also responsible for the increased absorption of lutein because, as mentioned before, this xanthophyll is very sensitive to the presence of lipids in the meal [31]. This could have been controlled by having a group of guinea pigs with added the same amount of MCT oil but not in the form of a nanoemulsion. However, the diet was 15% fat, which represents 2.55 grams of fat per day per guinea pig, since they ate 17 grams of food per day. With the nanoemulsion, which is 0.02% of the total diet and 11.2% MCT oil, this amount only increases by 0.04 grams of fat per day, which cannot

explain the significant increase of lutein absorption observed in the NANO group. Further studies with this preparation need to be done to clarify this issue.

4.6 Conclusions

For the results presented in this chapter, we conclude that the nanoemulsion used for this study did increase the bioavailability of lutein, as evidenced by a significantly higher concentration in all tissues when compared to control and in liver and plasma when compared to both control and PL groups. This suggests that nanoemulsions could be a promising approach to increase the biological effects of lutein in humans. Our results also suggest that this lutein nanoemulsion could be used as a supplement or as an ingredient in functional foods.

FIGURES

Figure 4.1 Concentration of lutein in plasma of guinea pigs fed no lutein (control), powdered lutein (PL) (3.5 mg/d), or lutein nanoemulsion (NANO) (3.5 mg/d) for 6 wks. Values are mean ± SD, n = 8. Bars without a common letter differ at P < 0.05

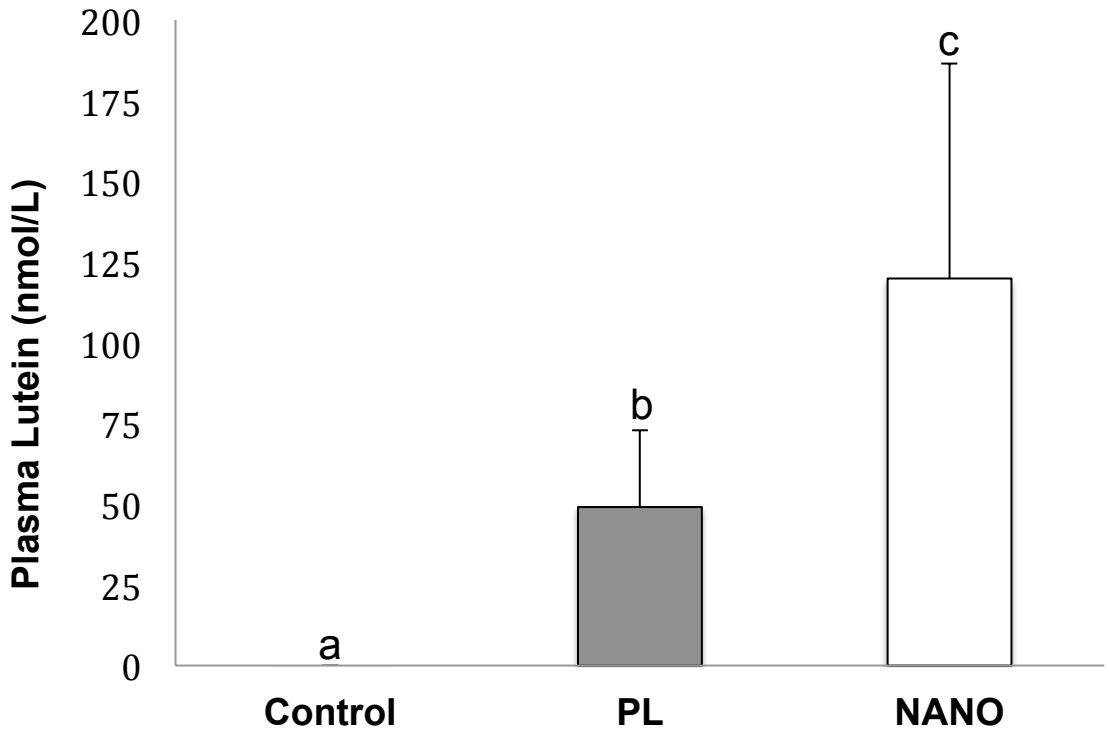


Figure 4.2 Concentration of lutein in livers of guinea pigs fed no lutein (control), powdered lutein (PL) (3.5 mg/d), or lutein nanoemulsion (NANO) (3.5 mg/d) for 6 wks. Values are mean ± SD, n = 8. Bars without a common letter differ at P < 0.05.

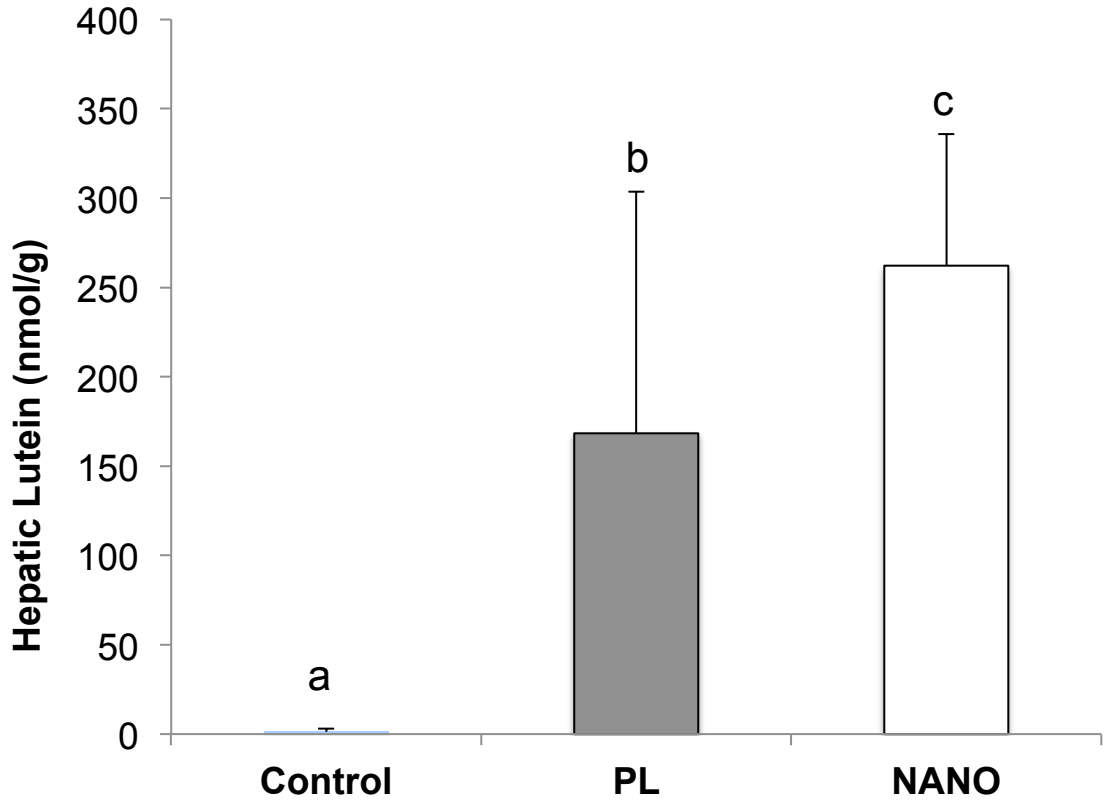


Figure 4.3 Concentration of lutein in eyes of guinea pigs fed no lutein (control), powdered lutein (PL) (3.5 mg/d), or lutein nanoemulsion (NANO) (3.5 mg/d) for 6 wks. Values are mean ± SD, n = 8. Bars without a common letter differ at P < 0.05.

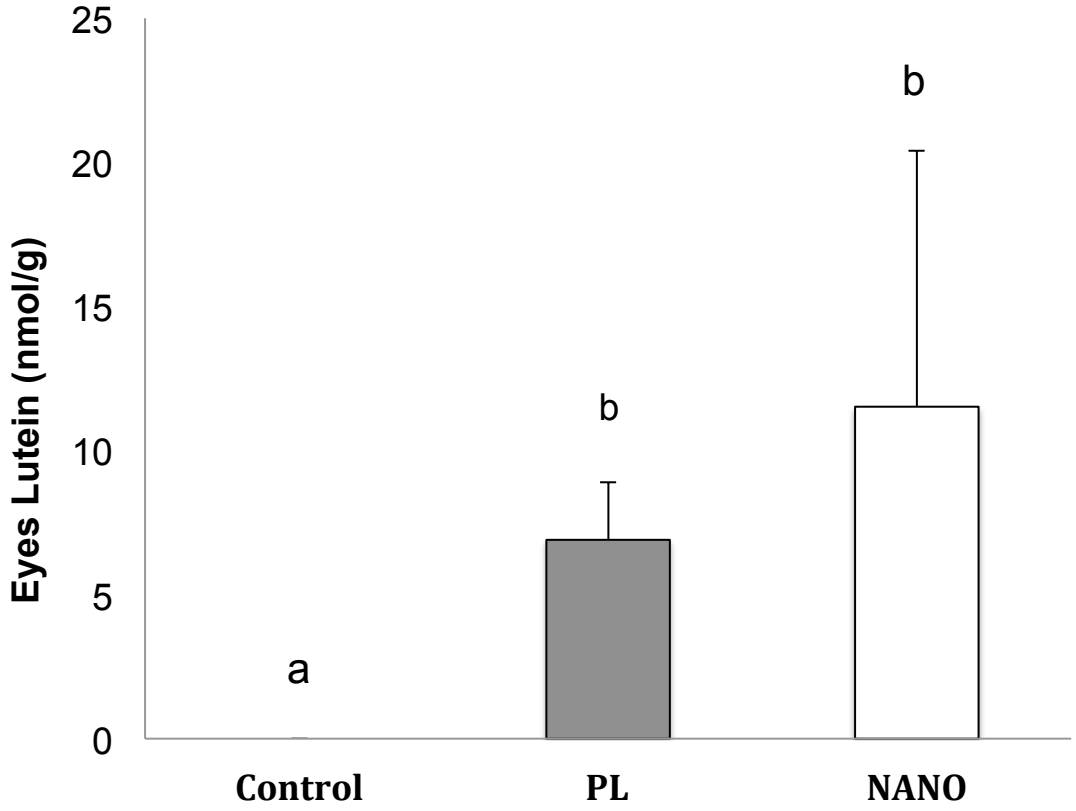
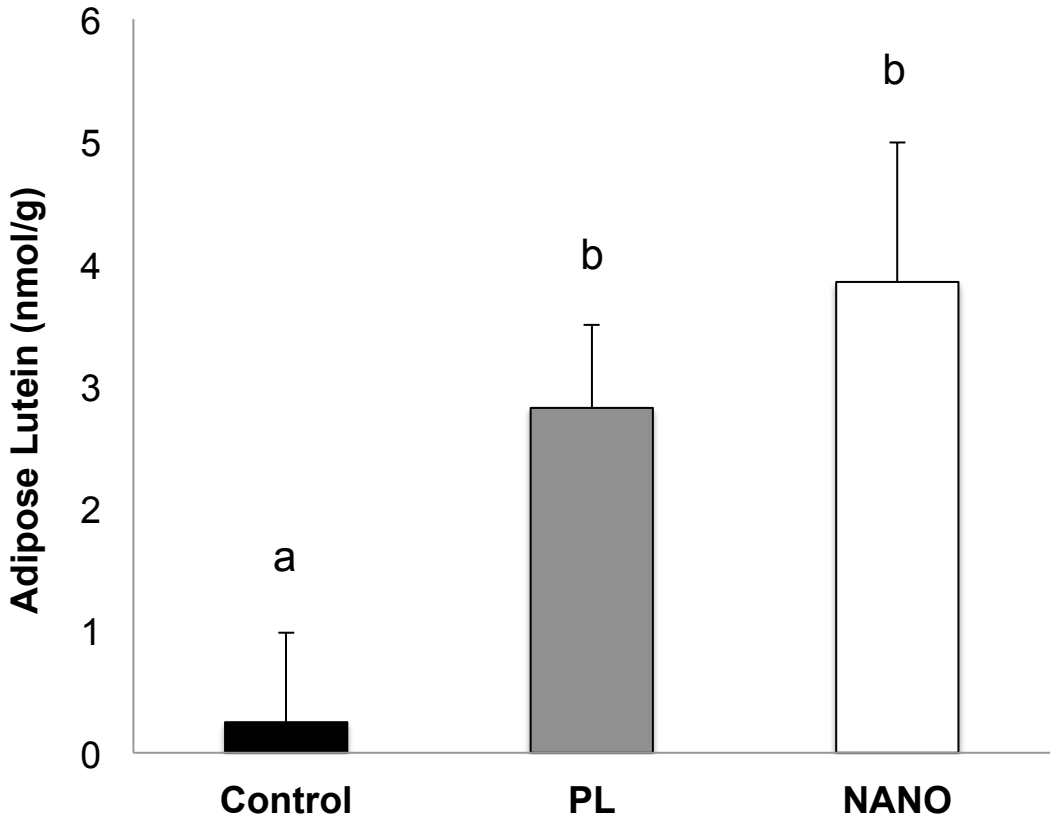


Figure 4.4 Concentration of lutein in adipose tissue of guinea pigs fed no lutein (control), powdered lutein (PL) (3.5 mg/d), or lutein nanoemulsion (NANO) (3.5 mg/d) for 6 wks. Values are mean ± SD, n = 8. Bars without a common letter differ at P < 0.05.



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Chapter 5

The effects of a nanoemulsion of lutein on cholesterol-induced hepatic damage

5.1 Background

The generation of reactive ROS plays an important role in producing liver damage. ROS disrupt lipids, proteins and DNA and moreover, ROS induce necrosis, apoptosis of hepatocytes and amplify the inflammatory response [1]. As mentioned previously, normal hepatic metabolism produces ROS, however, external factors such as drugs, alcohol, environmental pollutants and in this case, a cholesterol challenge also generate oxidative stress [2,3]. Kupffer cells, hepatic stellate cells and endothelial cells are particularly sensitive to oxidative stress-related molecules, including OxLDL [4]. Pro-inflammatory cytokines such as TNF-α can be produced in Kupffer cells induced by oxidative stress, and create an inflammatory response or even induce apoptosis [1,2].

Because the increasing prevalence of NAFLD, many natural dietary compounds have been widely studied for prevention and treatment of this disease. Among these, carotenoids such as lycopene, beta-carotene, astaxanthin and lutein have been studied [5–8]. The exact mechanisms of the protective effects of carotenoids in NAFLD are not completely understood but it is hypothesized that their potential relies on their antioxidant and anti-inflammatory properties [9].

The effects of lutein on cholesterol-induced liver damage has been tested previously in our laboratory by Kim *et al.* [7]. After feeding guinea pigs a hypercholesterolemic (0.25% (wt:wt) cholesterol) diet with or without 0.1% (wt:wt) lutein, equivalent to 3 mg/lutein/day, Kim *et al.* found that despite the expected injury induced by the cholesterol challenge in all animals, the ones supplemented with lutein had 43% lower hepatic FC than the

controls. Lutein fed guinea pigs also had lower protein concentration of TNF-α and lower NF-κB DNA binding activity than the control group, suggesting that lutein exerts anti-inflammatory effects in hepatic tissue [7].

Our hypothesis for this project is that a nanoemulsion of lutein would increase the absorption of this xanthophyll, making it more effective than regular powdered lutein in reducing cholesterol-induced liver damage with an intake of 3.5 mg/lutein per day in guinea pigs.

5.2 Materials and methods

5.2.1 Liver Enzymes: alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were measured using a Cobas C 111 analyzer (Roche Diagnostics, Indianapolis, IN). This instrument uses direct enzymatic reactions and photometric detection.

5.2.3 OxLDL: Tissue total protein was extracted using RIPA buffer and total protein concentration of the lysates were determined using the BCA Protein Assay Kit (Cell Signaling Technologies Inc, Beverly, MA) [10]. Using the same concentration of protein for all samples hepatic OxLDLs were measured with a specific guinea pig OxLDL ELISA kit (MyBioSource, Inc, San Diego California) according to manufacturers instructions.

5.2.4.Cholesterol Concentration in Hepatic Tissue: 10 mg of liver for each sample was homogenized with 200 µL of chloroform/isopropanol/MP40 (7:11:01). The slurries were centrifuged and the supernatant was dried at 50°C overnight. The quantification for total

(TC) and free cholesterol (FC) were done with enzymatic assay kits (Wako Diagnostics, CA) according to Carr *et al.* [11] The esterified cholesterol (EC) fraction was calculated manually by subtracting FC from TC. [12]

5.2.5 Histologic Evaluation: Small pieces of liver tissue were immersed in 10% buffered formalin. Formalin-fixed livers were paraffin embedded and 3–5 μm sections were stained with hematoxylin and eosin. Histologic evaluation was performed on two separate occasions by a veterinary pathologist blinded to the treatments, using a semi-quantitative scoring system to assess the severity of hepatic steatosis and inflammatory cell infiltration in 10 microscopic fields examined at 200X [13]. The following criteria was used for scoring hepatic steatosis: grade 0, no fat; grade 1, fatty hepatocytes occupying <33% of the hepatic parenchyma; grade 2, fatty hepatocytes occupying 33–66% of the hepatic parenchyma and grade 3, fatty hepatocytes occupying >66% of the hepatic parenchyma [13,14].

5.2.6 Inflammatory Cytokine Concentration in Liver: Tissue total protein was extracted using RIPA buffer and total protein concentration of the lysates were determined using the BCA Protein Assay Kit (Cell Signaling Technologies Inc, Beverly, MA) [10]. Using the same concentration of protein for all samples, the following cytokines were measured using Luminex technology (Luminex MAGPIX System, Austin, TX) with the MILLIPLEX MAP Rat Cytokine Immunoassay kit (Millipore corporation, Charles, MO, USA): IL-1β, IL-6, IL-10, IFN-γ, MCP-1, and TNFα, as previously described [15].

5.2.7 Gene expression

Primers design: Specific primers (**Table 5.1**) for *Cavia porcellus* were designed with the National Center for Biotechnology Information website.

Nucleic Acid Extraction: Liver tissue (~100mg) were homogenized and RNA was extracted with the Trizol® (Thermo Fischer Scientific, Waltman, MA) method [16] . cDNA was synthesized and amplified and quantified using real time PCR using a Bio-Rad CFX96 Real-Time system (BioRad, Hercules, CA).

RT-qPCR: Housekeeping genes were analyzed for threshold cycles (Ct). Each gene of interest was analyzed for relative expression using the same reaction mix and then standardized the gene of interest's Ct to the geometric mean of the Ct of the housekeeping genes using the $2^{-\Delta\Delta Ct}$.

5.2.8 Statistical Analysis

Differences between groups were analyzed by one-way ANOVA and Fisher's LSD post hoc analysis. All analyses were conducted on SPSS for Windows, Version 20 (IBM Corp.)

5.3 Results

5.3.2 Liver enzymes

For liver enzymes, there was no difference between AST activity between groups; however, ALT was significantly lower in both the PL and the NANO groups when compared to the controls **(Figure 5.1)**

5.3.3 Hepatic lipids, Liver Inflammation and Steatosis

Hepatic total cholesterol and esterified cholesterol concentrations were lower in the NANO group **(Table 5.2)**. There were no differences in hepatic cytokines IFNγ, IL-6, IL-10, MCP-1 or TNFα)among groups except for IL-β, which was lower in both lutein (32.3 ±9.1 ng/g) and NANO groups (26.4±7.0 ng/g) when compared to controls (45.4±16.8 ng/g) ($P < 0.05$) **(Table 5.3).**

Histologic evaluation of steatosis showed that the NANO group had a lower steatosis score (1.8±0.83) when compared to both lutein (2.75±0.46) and control (2.6±0.52) ($P < 0.05$). This attenuated accumulation of liver lipids in the NANO group is also shown in **Figure 5.2** where the arrows show lipid droplets in the hepatic tissue**.** A marked reduction in the degree of steatosis was observed in the livers from the guinea pigs fed the lutein nanoemulsion while there was no apparent difference between the groups fed powdered lutein (PL) or no lutein (control) **(Figure 5.3).**

5.3.4 Oxidative stress markers

Oxidative stress was assessed by the measurement of OxLDL in hepatic tissue. As shown in **Figure 5.4**, both PL and NANO groups showed a lower concentration of OxLDL (9.1±4.3 ng/mg and 12.3±4.3 ng/mg, respectively) when compared to the control group (21.7±14.4 ng/mg).

5.3.5 Hepatic gene expression

According to the results from qRT-PCR, the relative gene expression of acyl-coenzyme A:diacylglycerol acyltransferase 2 (Dgat2) was lower in the NANO group when

compared to the control group ($P<0.05$). In terms of cholesterol metabolism related genes, guinea pigs from the PL group, but not the NANO group, showed a higher expression of the sterol regulatory element-binding protein 2 (Srebpf2). Figure 5.5. No other differences in gene expression were observed among groups in the tested genes.

5.4 Discussion

5.4.1 Liver enzymes

ALT and AST are enzymes involved in amino acid and protein metabolism that are commonly used as markers for liver damage because when there is hepatic injury, these enzymes leak to the bloodstream leading to their detection in plasma [17–19]. Previous studies have shown that lutein can decrease cholesterol induced liver damage in guinea pigs [7] and in this study, a similar effect was observed for ALT, but not AST, in both PL and NANO groups (table 2). The reduction in only ALT can be explained by the fact that both enzymes are highly concentrated in the liver although AST is also diffusely present in the heart, skeletal muscle, brain and kidneys suggesting that elevations in plasma ALT is more specific for liver injury [17]. This hepato-protective action of lutein may be due to this carotenoid's capacity to scavenge reactive oxygen radicals and thus, decreasing oxidative stress in the hepatocytes, which can be translated into less ALT leakage and activity [20].

5.4.3 Liver lipids and Histologic evaluation

Previous studies have shown that a cholesterol challenge leads to hepatic steatosis in animal models [21,22]. As mentioned before, hepatic steatosis is mostly an accumulation of TG and fatty acids in the hepatocytes; however, lipidomic studies have shown an increase in hepatic FC concentration can accelerate the development of NAFLD and NASH [23]. Mechanistically, this FC may activate macrophages and also contribute to lipotoxicity and liver injury [24,25]. Hepatic cholesterol accumulation can be attributed to an up-regulation of LDL-R via activation of sterol regulatory element binding protein 2 (SREBP-2), reduced biotransformation to bile acids, and suppression of canalicular pathways for biliary cholesterol and bile acid excretion [24].

In the current study, the NANO group showed a lower concentration of hepatic TC and CE, however, no differences were found in FC concentrations. In the histological evaluation, nevertheless, the livers of the NANO group were considered to have lower steatosis score which is paradoxical because hepatic steatosis means excessive deposition of fat in the liver, and in our results this only coincides with the results reported for TC and EC [26].

Regarding the differences found in cholesterol, it is possible that in the NANO group, the excess cholesterol was mobilized to extra-hepatic tissues via VLDL, which could explain both the reduction of cholesterol in the hepatocytes and the higher number of VLDL particles found in plasma (reported in the next chapter).

5.4.4 Hepatic inflammatory markers

Besides being a potent antioxidant, lutein has shown to exert anti-inflammatory effects [7,27]. Although many cytokines were evaluated, significant differences were only observed in the case of IL-1β. Both lutein treatments resulted in a lower concentration of this cytokine. IL-1β is an important marker for hepatic damage because in liver disease, IL-1β promotes the recruitment of inflammatory cells to the liver and activates hepatic stellate cells (HSCs), which contributes to fibrosis, an important characteristic of the progression from hepatic steatosis to steatohepatitis [28].

5.4.5 OxLDL

OxLDL is a mixture of lipoproteins with heterogeneous modifications such as oxidation of phospholipids, modification of apolipoprotein B (apoB) with malondialdehyde and aggregation of apoB [29]. These particles, in addition to being a functional biomarker for atherosclerosis, exhibit pro-inflammatory, immunogenic, apoptotic, and cytotoxic activities which can affect other organs and be a risk factor for other diseases, including NAFLD [30].

When LDL become oxidized, these particles display structures that are similar to pathogen-associated epitopes that increases their uptake by macrophages through SR-A and CD36 [31]. This uptake has been widely documented for circulating macrophages, but also it has been reported that OxLDL is cleared from circulation by Kupffer cells, which are resident macrophages in the liver [29]. Specific membrane receptors for OxLDL have also been reported in HSCs and liver endothelial cells [4].

In vitro studies show that OxLDL particles are further trapped in the lysosome compartment due to poor degradation, leading to presence of cholesterol crystals which can activate the inflammasome and induce the activation of NF-κB signaling [32,33]. Therefore, this OxLDL derived lysosomal lipid accumulation can been linked to hepatic inflammation, liver fibrosis and the progression of NASH [4,32].

5.4.5 Gene Expression

Diglyceride acyl transferase (DGAT) is the enzyme that catalyzes the final step in TG synthesis by adding a free fatty acid to sn-1,2- diacylglycerol [34,35]. The enzyme has two isoforms, DGAT1 and DGAT2, however, DGAT2 is the one primarily expressed in the liver [36]. Studies with animal models have suggested that this enzyme plays an important role in the development of hepatic steatosis [23]. Cheol *et al.* [36] showed in a rat model of diet-induced NAFLD, that targeting DGAT2 with anti-sense oligonucleotides not only improves hepatic steatosis, but also hepatic insulin signaling, and in vivo hepatic insulin sensitivity. The treated animals also showed decreased Sterol regulatory element-binding transcription factor 1 (SREBP1c)-mediated lipogenesis and increased hepatic fatty acid oxidation [36]. Similar results were obtained by Yu *et al.*[37] in a study done with high-fat diet-induced obese mice and ob/ob mice. The antisense oligonucleotide (ASO) reduced DGAT2 messenger ribonucleic acid (mRNA) concentration by more than 75% in the liver, which resulted in decreased DGAT activity in this tissue. The treatment did not cause significant changes in body weight, adiposity,

metabolic rate or insulin sensitivity; however, it caused a significant reduction in hepatic triglyceride content and improved hepatic steatosis in both models, caused not only by a decrease in triglyceride synthesis but also an increase in fatty acid oxidation [37]. In our study, the NANO group showed a significant reduction of hepatic gene expression of DGAT2 when compared to controls and this can explain the differences observed in steatosis score. Surprisingly, these data do not correlate with hepatic TG concentration, since there was no difference among groups. This could be because hepatic steatosis was not induced by a high fat diet, so the differences were not as abrupt to detect significant differences in TG accumulation. It is believed that in NAFLD progression, TG accumulation occurs initially as the “first hit” and the progression to more advanced stages of NAFLD, including inflammation and fibrosis (NASH), require a “second hit”. Dietary cholesterol could be this second hit, as it exacerbates hepatic macrophage infiltration, apoptosis, and oxidative stress. Therefore, cholesterol could be more related to NAFL progression to NASH than NADL induction [22,38,39]. In guinea pigs, however, a cholesterol challenge like the one we used for this study has shown to also induce the first hit, as it significantly increases the TG concentration in hepatic tissue [21,40].

As the experiment was designed with a cholesterol challenge, and the liver is a key organ for cholesterol metabolism [41], one of the main interests of this part of the study was the measurement of cholesterol metabolism-related genes.

Intracellular cholesterol can be obtained through two pathways: uptake of LDL-C mediated by specific receptors (LDL-R) and by endogenous synthesis, mediated by the

mevalonate pathway, of which HMGCR is the rate-limiting enzyme [42]. An increased synthesis or a decreased uptake could explain the interesting findings in plasma lipoproteins (reported in the next chapter). Further, NAFLD has been associated with increased HMGCR expression and decreased phosphorylation [43]. In this study no differences in HMGCR mRNA expression was observed, however, the regulation of this enzyme is not only transcriptional, but also post-translational. High AMP:ATP ratio stimulates AMP-activated protein kinas, which phosphorylates HMGR at a conserved serine in the active site, inhibiting HMGR activity [44].

The inducible degrader of the LDL receptor (IDOL), also known as myosin regulatory light chain interacting protein (MYLIP) is an E3 ubiquitin ligase that mediates ubiquitination and degradation of the LDL-R limiting cholesterol uptake. IDOL is reported to be active in macrophages, adrenals, intestine and liver and play a role in the tissue response to changing cholesterol concentration [45,46]. Inhibition of IDOL could reduce plasma LDL-C by increasing LDL receptor density in the plasma membrane [46].

Srebf2, the gene that encodes for SREBP-2 which is known to up-regulate genes involved in cholesterol biosynthesis and uptake [47] was higher in the PL group compared to both NANO and controls. As mentioned before, SREBP-2 activation can lead to an up-regulation of LDL-R, however, this was not observed in any of the groups. Further, no significant differences were observed in the cholesterol content in the PL group. It is important to note that the SREBP transcription factors are relatively weak

activators of gene expression alone, and these usually require the cooperation of other co- factors [48].

There was no difference in cluster of differentiation 36 (CD36) or cluster of differentiation 68 (CD68), CETP, Liver X receptor alpha (LXR-a) (encoded by the *NR1H3* gene), SREBF1 or IL-β.

5.5 Strengths and limitations

Among the strengths of this study is the use of guinea pigs as animal model. Guinea pigs not only resemble humans in lipoprotein metabolism, but also in the synthesis, storage, and catabolism of cholesterol in the liver [40]. This peculiarity is relevant when studying hepatic steatosis and NAFLD, because cholesterol accumulation in the hepatocytes is reported to be a key molecule in the transition to NASH [49]. Moreover, guinea pigs have shown to respond to dietary interventions, including carbohydrate restriction [21,50], dietary fiber [51] and most relevant for this study, lutein supplementation [7]. Powdered lutein has been shown to be protective against cholesterol-induced injury, which gave us a baseline to design this study adding the nanoemulsion.

One limitation is that because this study design did not have a vehicle control group, it can be speculated that that the antioxidant effects of lutein could have acted synergistically with vitamin E in case this was released from TPGS. However, as previously mentioned, no vitamin E was found in plasma of the NANO group, which

suggest that the antioxidant effects observed are attributable to lutein only. Moreover there was no difference in hepatic OxLDL concentration between PL and NANO groups, suggesting that there was no synergistic effect between lutein and vitamin E in the NANO group.

Another limitation would be the number of animals used in each group. There were many trends observed that may have reached significance if more statistical power was achieved [52].

5.6 Conclusions

The results for this part of the study show that a nanoemulsion of lutein is more effective that the same dose of powdered lutein in decreasing cholesterol-induced liver damage, specifically hepatic steatosis. However, no significant effect was observed in pro-inflammatory cytokines. These results indicate that a higher concentration of lutein in the liver could give a greater protection against hepatic cholesterol accumulation but does not cause significant change in inflammation caused by a cholesterol challenge.

TABLES

Table 5.1 List of designed primers for *Cavia Porcellus* for q-RT PCR

Gene	Protein	5'-Forward Primer-3'	5'-Reverse Primer-3'
SREBF2	SREBP2	CTGAACGGGACAGCAGAGAG	CAAACTGCAGCATCTCGTCG
SREBF1	SREBP1	GGTGAGGTTTCCCGTTTTGC	GAGGTGTCGGGACTTTGAGG
NR1H3	LXRα	AAAGACCTCATGTCCTGCCG	CAACCCGCAAAGTGACAGTG
MYLIP	IDOL	AGTCTGGGAACAGCATCGTG	GTTTCGGTGATGGCTCGGTA
LDLR	LDLR	ACGTCAATGGCAGAGACAGG	CCTGTAAGGCGGTTGACACT
IL-1β	IL-1β	TCCAGCGGATCTTCATTGCT	TGCAGCTTGATCCCCTCATC
HMGCR	HMGCR	CAGTGTGACTGTTCCCACGA	CAGTGTGACTGTTCCCACGA
GAPDH	GAPDH	GTCTGGCAAAGTGGATAT	GGTAGAATCATACTGGAACA
DGAT2	DGAT2	TCCTACCGGGATGTCAACCT	TTGTCTGGGTAGCGCACAAT
CETP	CETP	TGACGGGGGACAAGTTCAAG	TCACGGAAGAACTGACCACG
CD68	CD68	CACCACCACCAATCATGGGA	ACGGTGGTAGCATTTCCAGG
CD36	CD36	ATCTCTTTCGTGCAGCCCAA	GCCAGGTTGAGGACTGTGAA
ACTB	β-Actin	CCTCTATGCCAACACAGTGC	GTACTCCTGCTTGCTGATCC

Table 5.2 Concentration of hepatic total cholesterol (TC), free cholesterol (FC) and cholesterol esters (CE) of guinea pigs fed a hypercholesterolemic diet with no lutein (control) powdered lutein (PL) (3.5 mg/d) or nano-lutein (NANO) (3.5 mg/d) for 6 wk. [1]

	Control	PL	NANO
TC (mg/g)	2.15±0.28[a]	2.05±0.58[a]	1.50±0.40[b]
FC (mg/g)	0.50±0.09	0.40±0.21	0.38±0.12
CE (mg/g)	1.63±0.26[a]	1.50±0.39[ab]	1.18±0.42[b]

[1] Values are means ± SD, n=8. Values in the same row with different superscripts are significantly different at P< 0.05.

TABLE 5.3. Concentration of hepatic inflammatory cytokines of male guinea pigs fed control, powdered lutein (PL) (3.5mg/d) or a nanoemulsion of lutein (NANO) (3.5 mg/d) diets for 6 wk [1].

	Control	PL	NANO
IFN-γ (ng/g)	155 ± 21	163 ± 53	155 ± 35
IL-1β (ng/g)	45 ± 17[a]	32 ± 9[b]	26 ± 7[b]
IL-6 (ng/g)	118 ± 35	98 ± 26	108 ± 17
IL-10 (ng/g)	445 ± 40	483 ± 70	469 ± 98
MCP-1 (ng/g)	31 ± 5	37 ± 10	32 ± 14
TNFα (ng/g)	39 ± 4.0	43 ± 13	40 ± 14

[1] Values are means ± SD, n=8; Values in the same row with different superscripts are significantly different at $P < 0.05$

FIGURES

Figure 5.1 Activity of liver enzymes Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) of guinea pigs fed a hypercholesterolemic diet with no lutein (control) powdered lutein (PL) (3.5 mg/d) or nano-lutein (NANO) (3.5 mg/d). Values are mean ± SD, n=8. Bars without a common letter differ at P < 0.05.

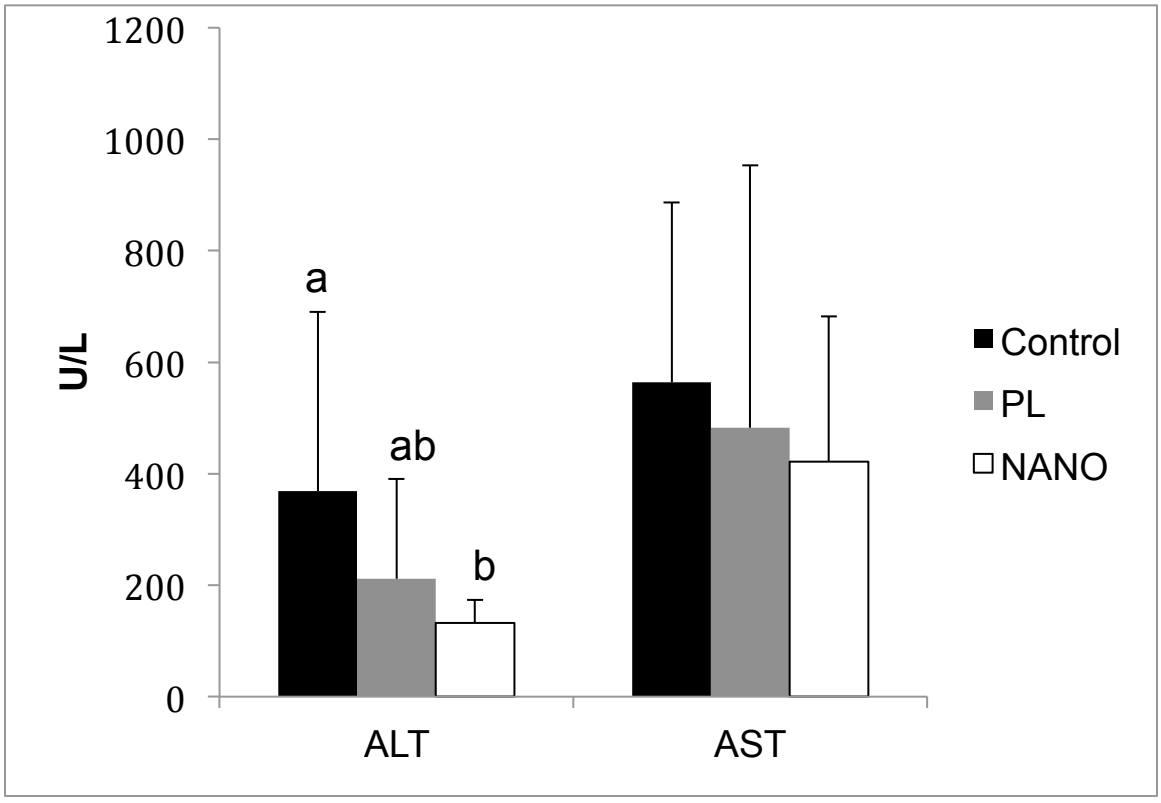


Figure 5.2 Hepatic tissue histological evaluation of guinea pigs fed a hypercholesterolemic diet with no lutein (control, panel A) powdered lutein (PL, panel B) (3.5 mg/d) or nano-lutein (NANO, Panel C) (3.5 mg/d). Red arrows indicate lipid accumulation

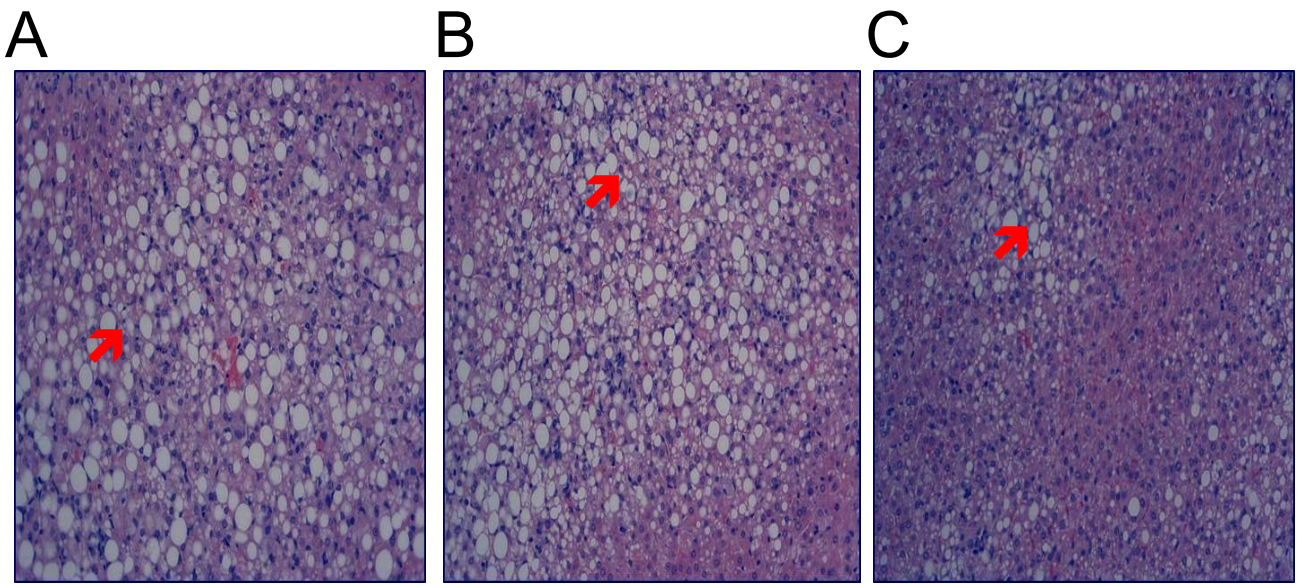


Figure 5.3 Hepatic steatosis score of guinea pigs fed a hypercholesterolemic diet with no lutein (control) powdered lutein (PL) (3.5 mg/d) or nano-lutein (NANO) (3.5 mg/d). Values are mean ± SD, n=8. Bars without a common letter differ at P < 0.05.

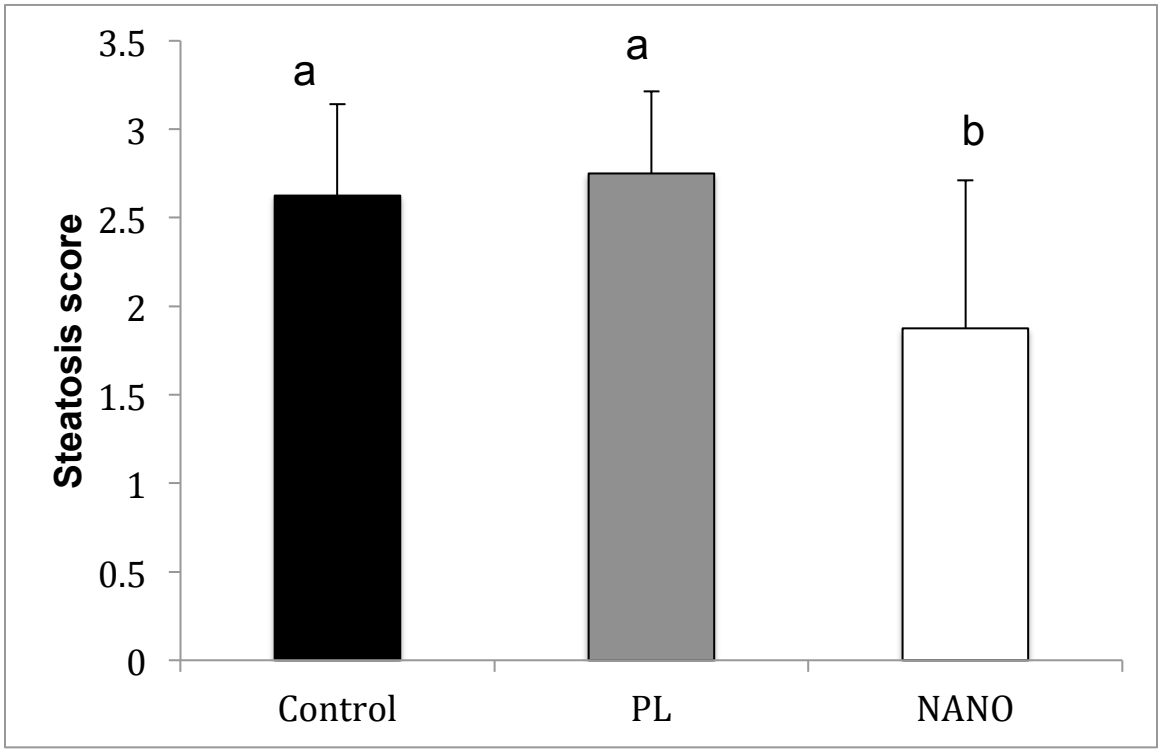


Figure 5.4 Concentrations of OxLDL in hepatic tissue of male guinea pigs fed a high cholesterol diet with no lutein (control), powdered lutein (PL) (3.5mg/d), or a nanoemulsion of lutein (NANO) (3.5mg/d) for 6 wks. Values are mean ± SD, n=8. Bars without a common letter differ at P < 0.05.

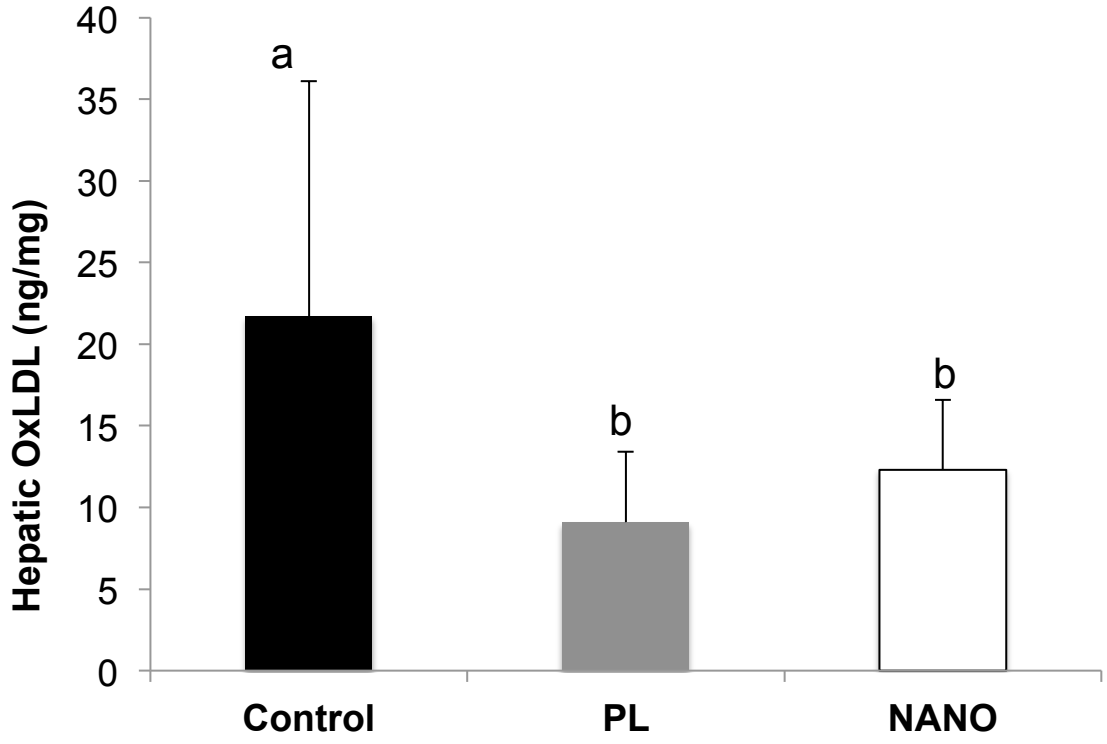
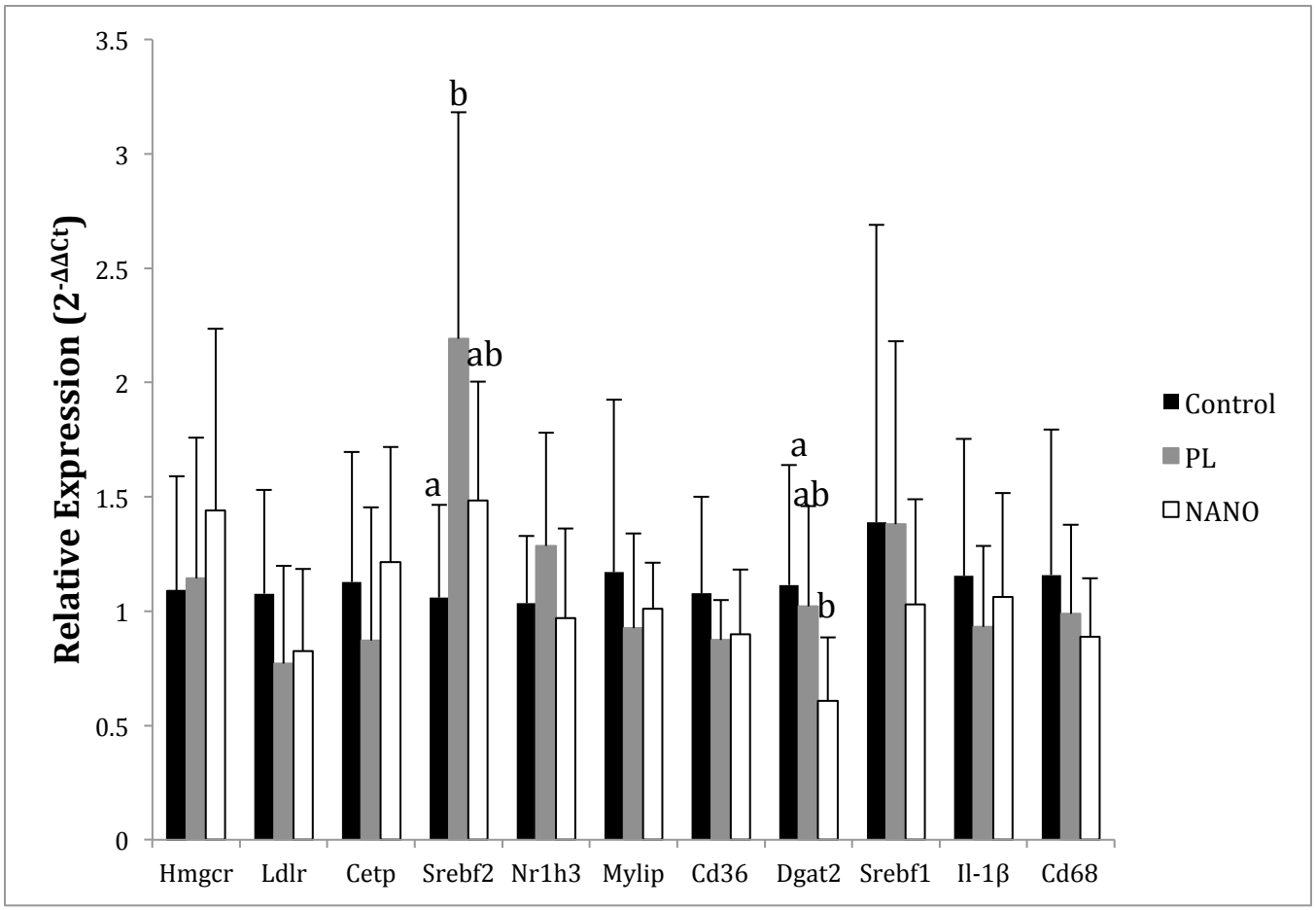


Figure 5.5 Hepatic relative Gene expression of male guinea pigs fed a high cholesterol diet with no lutein (control), powdered lutein (PL) (3.5mg/d), or a nanoemulsion of lutein (NANO) (3.5mg/d) for 6 wks. Values are mean ± SD, n=8. Bars without a common letter differ at P < 0.05.



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Chapter 6

The effects of a nanoemulsion of lutein on plasma lipoproteins and adipose tissue inflammation

6.1 Background

The insolubility of cholesterol and TG in plasma requires that they are transported in lipoproteins, which are distinguished from each other by size, density, electrophoretic mobility, composition and function [1]. Understanding lipoprotein metabolism has important clinical implications because altered lipoprotein profiles are considered a key risk factor for numerous chronic diseases such as CVD, MetS, NAFLD and T2DM, among others [2–5].

Early work suggested that dietary cholesterol increased plasma total cholesterol concentrations in humans [6]. However, it is now well established that the response to dietary cholesterol varies among individuals [7]. Besides, not only may dietary cholesterol raise circulating cholesterol concentrations, but also fatty acid or carbohydrate-induced cholesterol biosynthesis can contribute to this phenomenon [8,9]

The guinea pig has been shown to be a suitable model for investigating lipoprotein profile changes mediated by dietary factors, including cholesterol challenges [9–11]. The great advantage of these animals is that their cholesterol metabolism is the closest to the human situation in small laboratory animals [12] which makes them ideal models to understand molecular mechanisms and alterations of metabolic pathways induced by dietary interventions [10,13]

White adipose tissue has traditionally been considered as an inert tissue mainly devoted to fuel storage in the form TG, however, it is now recognized that this tissue has very

important roles in homeostasis, energy balance, glucose metabolism, immunity and inflammation [14–16].

In addition to adipocytes, the adipose tissue includes nerves, immune cells and connective tissue. This complexity not only affects structure but also functionality of the adipose tissue. Nowadays adipose tissue is considered a very active endocrine organ which secrets several key hormones, such as leptin and adiponectin, but also many other bioactive substances called adipokines [17]. These adipokines include pro-inflammatory cytokines such as TNFα, IL-6, and MCP-1[17].

An important feature of inflammation is infiltration of immune cells such as neutrophils, eosinophils and macrophages. Macrophage infiltration in adipose tissue has recently been described in obese conditions in rodents and humans [18]. This infiltration alters the production and release of pro-inflammatory cytokines that can trigger insulin resistance and the hepatic production of acute inflammatory molecules like Serum Amyloid A and C-reactive protein, creating a loop of systemic inflammation [19].

Dietary cholesterol has been reported to exacerbate adipose tissue inflammation in several animal models [19,20], suggesting that high dietary cholesterol can lead to adipocyte dysfunction and contribute to systemic inflammation [21].

In addition to their scavenging function toward reactive oxygen species (ROS), there is growing evidence that carotenoids may also act via more indirect pathways. This

indirect route may include interactions with cellular signaling cascades, such as NF-κB, MAPK, or c (Nrf2) [22].

It was our hypothesis for this part of the study that a nanoemulsion of lutein would be more effective than the same dose of regular powdered lutein in ameliorating cholesterol induced dyslipidemia and inflammation in the adipose tissue.

6.2 Materials and Methods

6.2.1 Fasting Plasma Lipids: TC, HDL HDL-C, and TG, were measured using a Cobas C 111 analyzer (Roche Diagnostics, Indianapolis, IN). This instrument uses direct enzymatic reactions and photometric detection. LDL-C was calculated using the Friedewald equation [23].

6.2.2 Lipoprotein Subfractions and Size: NMR spectroscopy was performed on a 400-MHz NMR analyzer (Bruker BioSpin) as previously described [9]. This method provides the concentrations of large, medium and small VLDL, LDL and HDL particles [24,25].

6.2.3 OxLDL: Plasma OxLDLs were measured with a specific guinea pig OxLDL ELISA kit (MyBioSource, Inc, San Diego California) according to manufacturers' instructions.

6.2.4 Inflammatory Cytokine Concentration in the adipose tissue:

Tissue total protein was extracted using RIPA buffer and total protein concentration of the lysates were determined using the BCA Protein Assay Kit (Cell Signaling

Technologies Inc, Beverly, MA) [26]. Using the same concentration of protein for all samples, the following cytokines were measured using Luminex technology (Luminex MAGPIX System, Austin, TX) with the MILLIPLEX MAP Rat Cytokine Immunoassay kit (Millipore corporation, Charles, MO, USA): IL-1β, IL-6, IL-10, IFN-γ, MCP-1, and TNFα, as previously described [27].

6.2.5 Histologic evaluation

Small pieces of adipose tissue samples were immersed in 10% buffered formalin. Formalin-fixed adipose were paraffin embedded and 3–5 μm sections were stained with hematoxylin and eosin. A veterinary pathologist blinded to the treatments performed histologic evaluation on two separate occasions. A low magnification screen of the entire tissue was assessed for evidence of abnormality and crown-like structures and signs of inflammation were quantified in 10 fields at 200X.

6.2.6 Statistical Analysis:

Differences between groups were analyzed by one-way ANOVA and Fisher's LSD post hoc analysis. Pearson correlations were calculated between plasma lutein and plasma lipoproteins. $P < 0.05$ was considered to be significant. All analyses were conducted on SPSS for Windows, Version 20 (IBM Corp.)

6.3 Results

6.3.1 Plasma Lipids, Lipoprotein Size and Subfractions

The NANO group showed significantly higher concentrations of plasma TC, LDL-C and HDL-C compared to the other treatments($P < 0.001$) (**Figure 6.1**).

Total VLDL and all its subfractions (large, medium and small) were highest in the NANO group ($P < 0.05$) (**Table 6.1**). Similarly, total LDL, IDL, large and small LDL were highest in the NANO group. In the case of HDL, the total HDL was higher in the NANO compared to the other groups in agreement with highest concentrations of small HDL. In contrast, the large HDL was also lowest in the NANO group (**Table 6.1**). Significant correlations were found between plasma lutein and LDL-C **(Fig 6.2, Panel A)**, HDL-C **(Fig 6.2, Panel B**), number of LDL particles **(Fig 6.2, Panel C)** and number of HDL particles **(Fig 6.2, Panel D).**

6.3.3 Oxidative stress markers in plasma

Guinea pigs from the NANO group showed a significantly lower oxidation in LDL particles in plasma (2.8±0.95 ng/mL) when compared to PL (6.1±4.4 ng/mL) or control (7.9±2.1ng/mL) (**Figure 6.3**).

6.3.3 Cholesterol Accumulation and Inflammatory Markers in Adipose Tissue

TC and FC concentrations were higher in the NANO group compared to the other groups ($P < 0.05$), while EC concentrations were higher in the NANO and control groups, when compared to the PL group (**Table 6.2**).

The inflammatory markers IFN-γ and MCP-1 were higher in the NANO group compared to both PL and control groups; Similarly, IL-10, an anti-inflammatory cytokine, was also higher in the NANO group compared to other treatments (**Table 6.3**). Histologically, there was no differences in the adipose tissue morphology or in the presence of crown-like structures among groups.

6.4 Discussion

6.4.1 Plasma lipids and Lipopoproteins

The observed changes in plasma lipoproteins are unexpected and undesirable because they represent a more atherogenic lipoprotein profile [28]. An increase in LDL-C is traditionally seen as an independent atherogenic marker, however, if HDL-C is also increased, and the LDL/HDL ratio is unaltered there is no change in cardiovascular risk [3]. In the present study, LDL-C in the NANO group was three fold higher than the control group; while HDL-C was only near to double the concentration of the control group, therefore the LDL/HDL ratio with the nanoemulsion of lutein (3.3) was higher when compared to controls (2.5), indicating a shift towards an atherosclerotic risk. A similar outcome was observed for the TC/HDL ratio, also known as the Castelli ratio [3] since the TC concentration in the NANO group was 2.7 times higher than the TC concentration in the control group (**Figure 6.1**) yielding Castelli ratios of 3.7 in the control group and 4.4 in the case of the NANO group.

As previously stated, given the lipophilic nature of carotenoids, they are transported in lipoproteins once they are absorbed in the gut [29]. Lutein, specifically, is packed into

chylomicrons and transported via the lymphatic system to the liver, where it can be stored or further packed into VLDL to be transported throughout the body [30]. In addition to the chylomicron pathway, recent studies have shown that lutein could be incorporated into HDL directly from the basolateral membrane of the enterocyte via an ABCA1/ApoA1 pathway [31]. Independently of the absorption pathway, an important characteristic of xanthophylls is that they are mainly transported in HDL particles [32–35], whereas carotenes are preferably carried in LDL particles [34,36]. In the Los Angeles Atherosclerosis Study, it was shown in a cohort of 269 adult women and 304 men that plasma lutein concentration was positively correlated to HDL-C concentration [37]. Blesso *et al.* [35], showed that egg (a natural source of high bioavailable lutein) consumption in patients with metabolic syndrome increased not only the concentration but the size of HDL particles, which also had an increase of carotenoid content [35] Due to greater surface area, larger HDL particles are more suitable for xanthophyll transport [35]. These larger HDL particles are also hypothesized to be more anti-atherogenic [35,38].

It is important to note that lutein is also carried by LDL, and some authors have suggested that an increase in lutein concentration in the body would lead to an increase in both lipoproteins. As reported by Renzi *et al.*[34] and Waters *et al*. [39] circulating levels of xanthophylls lutein and zeaxanthin are positively correlated with both serum HDL and LDL fractions [34,39].

The same correlations were found in the present study, as lutein concentration in plasma is positively correlated not only with LDL and HDL cholesterol concentration, but also with the number of LDL and HDL particles. This situation could explain, partially, the higher concentrations of TC, LDL-C and HDL-C as well as of all lipoprotein subfractions. However, there was no difference between the powdered lutein and the control group, which suggests that a component of the nanoemulsion played a role in the alteration of the lipid profile seen in the NANO group. It could also be possible that the MCT oil used to emulsify the lutein for the NANO group had a negative impact on lipoprotein metabolism. It has been reported that a similar hypercholesterolemic effect was observed in humans. In a study with a double-blind, randomized, crossover design, 17 healthy young men were given 70 g of MCT oil (66% 8:0 and 34% 10:0) for 12 days resulting in 11% higher plasma TC, 12% higher LDL-C, 32% higher VLDL cholesterol and 22% higher plasma TG when compared to high-oleic sunflower oil (89.4% 18:1) [40]. Similar findings were reported by Carter *et al.* [41], who compared the lipid effects of a diet supplemented with either MCTs, palm oil, or high oleic acid sunflower oil in nine middle-aged men with mild hypercholesterolemia. Although palm oil is known to have hypercholesterolemic effects and MCT are popularly believed to be neutral, in Carter's study MCT oil increased the concentration of TC and LDL-C in a similar fashion to palm oil. Both palm and MCT oils resulted in significantly higher concentrations of TC and LDL-C when compared to oleic acid [41]. What makes MCT attractive is that they bypass the CM pathway and are sent directly to the liver via the portal vein where they can be rapidly catabolized as opposed to be stored. It is hypothesized, however, that the acetyl CoA end products of those fatty acids (8:0 and

10:0) oxidation are resynthesized into long-chain fatty acids [8]. The increased *de novo* lipid synthesis would lead to an increase in hepatic TG production and thereby to VLDL secretion, which can explain the elevation in both TC and TG in plasma of animal and human studies [40,41].

This hypothesis could explain why, although the PL group had significantly more lutein than the control group in plasma and liver, the shift towards an atherogenic lipoprotein profile was only observed in the NANO group. However, the dose used for this Tholstrup's trial was 70 grams of MCT, which is substantially higher than the one used in the nanoemulsion. However, there are not enough data about how much MCT is needed to have this effect in plasma lipoproteins.

The MCT oil could have also affected cholesterol absorption. Based on the results presented in the previous chapter, it is possible that the differences observed in plasma lipoproteins are not derived from an increased synthesis of endogenous cholesterol or decreased degradation of LDL in the NANO group. We can speculate that the increased cholesterol concentration in plasma lipoproteins is derived from dietary cholesterol that could have been absorbed more efficiently with the addition of MCT oil to the diet. This assumption is supported by a study done with C57L mice, where Wang *et al.* showed that dietary cholesterol is absorbed differently when different oils are used as vehicle. Wang reported that with MCT oil, cholesterol showed a higher percentage of absorption when compared to other vehicles such as corn and soybean oil, but less absorption when compared to olive oil or safflower oil [42].

Alternatively, it could be possible that the fact that lutein was given in a nanoemulsion could have caused the detrimental changes, because an important consideration of nano-technology is that materials in the nano-scale behave differently from the same materials in bulk, which means that a change in size could have harmful effects in biological systems with a higher potential to generate some sort of toxicity or undesired effect [43].

Overall, the results suggest that the use of this particular nanoemulsion formulation may be detrimental in terms of cardiovascular risk independently of lutein concentration. Further studies with the nano-formulation used in the present study are needed to evaluate the effects of the different components of the nanoemulsion on lipoprotein metabolism.

6.4.2 OxLDL

An important risk factor for atherosclerosis is the accumulation of cholesterol-rich, apoB-containing lipoproteins like LDL in the intima of arterial walls [44–46]. These sequestered lipoproteins are prone to be modified via oxidation, enzymatic and non-enzymatic cleavage or aggregation, which make these particles pro-inflammatory and pro-atherogenic [45]. For example, OxLDL are accumulated in macrophages transforming them into the cholesterol-rich foam cells which along with apoptotic cells, debris and cholesterol crystals form a necrotic core and cause luminal narrowing of

arteries [45–47]. For this reason, circulating OxLDL in plasma is a good predictor of atherosclerosis and associated risk factors, and many studies have found strong correlations between plasma OxLDL with the disease progression [48].

In this study, despite the fact that the NANO group had a considerable elevation of LDL-C and smaller LDL particles, we found the lowest concentration of OxLDL particles in plasma. These results point to a protective effect of lutein on the oxidation of LDL in spite of the higher concentrations of this lipoprotein. Similar effects of lutein have been reported previously. Kim *et al.* [27] conducted a study with cholesterol challenged guinea pigs (0.25 g cholesterol/100 g) randomly assigned to a non-purified diet or a diet with a supplementation of 3 mg per day of lutein for 12 weeks. While plasma LDL cholesterol did not differ between groups, OxLDL concentrations were decreased in both plasma and aorta of supplemented guinea pigs [27].

Dwyer *et al.* [37] studied the effects of lutein supplementation with apoE knockout mice, which develop severe atherosclerosis morphologically similar to the human situation. In this experiment, 10 mice were assigned to a chow diet (control) or a chow diet with added lutein (0.2% by weight) starting at 8 weeks of age. The results of this experiment show that lutein supplementation decreased LDL oxidation in a dose-dependent manner [37]. Similarly, in a dietary intervention with thirty-five women with metabolic syndrome and high plasma LDL following a Mediterranean-style low-glycemic-load diet for 12 weeks, the changes in plasma oxLDL concentration were inversely correlated with plasma lutein [49]. It is believed that this effectiveness has to do with lutein-rich HDL

particles, which could be more effective in preventing the oxidation of LDL particles, given the fact that this polar carotenoid is located in the surface of lipoproteins. [50]

6.4.3 Cholesterol accumulation in adipose tissue

Previous studies have shown that dietary cholesterol challenges induce cholesterol accumulation in adipose tissue in guinea pigs [51] and that this cholesterol accumulation increases macrophage infiltration and concentrations of inflammatory cytokines [21]. Given the anti-oxidant and anti-inflammatory properties of lutein, and the fact that the adipose tissue is a site of carotenoid storage [52] we hypothesized that a higher concentration of this carotenoid in the adipose tissue would have protective effects against cholesterol-induced damage.

In this study, however, despite showing a higher concentration of lutein than the control group, the NANO group also had a higher concentration of FC when compared to controls and of TC and CE when compared to PL. FC is toxic to cells and induces inflammatory responses such as activation of the inflammasome [45]. In cultured cells, excess cellular FC is transported to the plasma membrane where it induces a number of cellular responses, including cholesterol crystallization and cell death [53]. These results can be explained by the fact that although adipose tissue stores most of the free cholesterol in the body, cholesterol synthesis is low when compared to other cell types, which means that the cholesterol found in this tissue comes from plasma lipoproteins [54]. In this study, the lipoprotein profile of the NANO group was significantly altered,

and these circulating lipoproteins could have negatively influenced the cholesterol content of the adipocytes.

6.4.4 inflammatory markers in the adipose tissue

Consequent to the increased concentration of FC, we also found a higher concentration of pro-inflammatory cytokines IFNγ and MCP-1 in the adipose tissue of the NANO group when compared to other treatments. Interestingly, IL-10, a known anti-inflammatory cytokine [55] was also higher in this group.

A possible explanation for these unexpected results in the adipose tissue is that carotenoids, besides antioxidants, are also reported to act as prooxidants under high oxygen tension, high carotenoid concentration and imbalanced intracellular redox status [56]. Under high oxidative stress, while scavenging ROS, carotenoids get oxidized and generate highly reactive aldehydes that have been linked to oxidative stress similar to that caused by lipid peroxidation products *in vitro* and *in vivo* [57]. However, there is a lack of data reporting a plasma or tissue lutein concentration linked to these detrimental effects, and in animal studies, dosages of lutein significantly higher than the one used for this study have not reported adverse effects or indication of lutein toxicity [58].

It is more likely that the detrimental changes observed in the NANO group are caused by the altered lipoprotein profile and not by lutein *per se*. Hypercholesterolemia has been shown to cause adipose tissue dysfunction in obese and not obese subjects [51] and in the present study, the plasma concentrations of both TC and LDL-C were

probably too high to be counteracted by the dose of lutein consumed by the guinea pigs and this could be the cause of adipose tissue increased cholesterol accumulation and inflammatory response in the NANO group. Interestingly, no differences were observed in the histological evaluation of the adipose tissue among groups.

6.5 Strengths and limitations

A strength of this study is the use of the guinea pig as the animal model, because these animals mimic human hepatic cholesterol and lipoprotein metabolism [10,13].

The main limitation of this part of the study is, once again, the lack of an empty vehicle control. In this case, having a group eating chow with a nanoemulsion without lutein could have tested the speculation that a low dose of MCT oil consumed by the NANO GROUP could have caused such significant changes in the lipid profile.

Also, as previously stated, the detrimental changes observed in the adipose tissue are most likely caused by the detrimental changes in the lipoprotein profile, which interfered with the potential of lutein in ameliorating the inflammatory responses in this tissue induced by a cholesterol challenge. It would have been interesting to observe if there were changes in the mRNA expression of genes of interest in the adipose tissue, however, the RNA extracted for this essay was degraded and the assays could not be performed.

As mentioned before, OxLDL is a good predictor of atherosclerosis [59], and we found lower concentrations of this modified lipoprotein both in plasma and liver, however, there are no data regarding the aortas of the guinea pigs or any other atherosclerosis

risk markers. This is because hypercholesterolemic guinea pigs show NAFLD features before developing atherosclerosis, which usually takes 12 weeks [27,60]. A longer study design could have added valuable data about lutein and atherosclerosis risks to this project.

6.6 Conclusions

Based on our results, it can be concluded that the formulation of the nanoemulsion of lutein used in this study caused detrimental changes in lipoprotein metabolism and inflammatory markers in the adipose tissue of guinea pigs. We cannot be certain which component of the nanoemulsion caused so much damage in the NANO group. However, it seems that this particular nanoemulsion, although increasing the bioavailability of lutein as discussed in previous chapters, exacerbates the negative impact of a cholesterol challenge in the organism. Further studies are needed to understand the mechanisms underlying this observed phenomenon and until then, the use of this specific nanoemulsion is not recommended for humans.

TABLES

Table 6.1 Lipoprotein subfractions of male guinea pigs fed control, powdered lutein (PL) (3.5 mg/d), or lutein nanoemulsion (NANO) (3.5 mg/d) for 6 wk[1].

	Control	PL	NANO
Total VLDL (nmol/L)	44.7±14.7[a]	56.8±27.9[a]	125±37.9[b]
Large VLDL (60-100 nm)	1.0±0.9[a]	1.6±0.9[a]	4.1±1.9[b]
Medium VLDL (40-60 nm)	11±4.6[a]	19±9.8[a]	38±14.5[b]
Small VLDL (30-40 nm)	32±10[a]	36±18[a]	83±23[b]
Total LDL (nmol/L)	506±116[a]	620±199[a]	1,190±246[b]
Large LDL (23-30 nm)	109±28[a]	102±33[a]	155±61[b]
Small LDL (20.5-23 nm)	341±103[a]	424±142[a]	859±178[b]
Very small LDL (18-20.5)	274±20[a]	341±31[a]	685±41[b]
Total HDL (µmol/L)	1.4±0.7[a]	2.0±1.0[a]	3.6±1.5[b]
Large HDL (10-13 nm)	0.15±0.15[a]	0.17±0.07[a]	0.03±0.05[b]
Medium HDL (8.2-10 nm)	0.14±0.18	0.24±0.13	0.11±0.18
Small HDL (7.3-8.2 nm)	1.2±0.7[a]	1.6±0.9[a]	3.5±1.5[b]

[1] Values are means ± SD, n=8; Means in the same row without a common letter differ at P < 0.05

TABLE 6.2. Concentration of adipose tissue TC, FC and CE of guinea pigs fed a hypercholesterolemic diet with no lutein (control) powdered lutein (PL) (3.5 mg/d) or nano-lutein (NANO) (3.5 mg/d). [1]

	Control	PL	NANO
Total Cholesterol (mg/g)	10.16±1.51[ab]	9.20±0.78[a]	11.52±2.50[b]
Free Cholesterol (mg/g)	5.10±0.30[a]	5.51±0.80[ab]	6.30±1.30[b]
Cholesterol Esters (mg/g)	5.03±1.50[b]	3.68±0.93[a]	5.13±1.58[b]

[1] Values are means ± SD, n=8. Values in the same row with different superscripts are significantly different at $P < 0.05$.

Table 6.3 Concentration of inflammatory cytokines in adipose tissue of male guinea pigs fed control, powdered lutein (PL), or nanoemulsion (NANO) diets for 6 wk [1].

	Control	PL	NANO
IFN-γ (ng/g)	76 ± 12[a]	70 ± 10[a]	120 ± 22[b]
IL-1β (ng/g)	103 ± 24	111 ± 30	122 ± 23
IL-6 (ng/g)	39 ± 3	38 ± 4	39 ± 11
IL-10 (ng/g)	106 ± 25[a]	113 ± 35[a]	155 ± 31[b]
MCP-1 (ng/g)	19 ± 2[a]	20 ± 2[a]	40 ± 21[b]
TNFα (ng/g)	18 ± 3	18 ± 2	22 ± 2

[1] Values are means ± SD, n=8. Values in the same row with different superscripts are significantly different at $P < 0.05$

FIGURES

Figure 6.1 Lipid profile: triglycerides (TG), total cholesterol (TC), high density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LDL-C) concentrations of male guinea pigs fed the control, powdered lutein (PL) and lutein nanoemulsion (NANO) diets (3.5mg/d) for 6 wks. Values are mean ± SD, n=8 per group. Bars without a common letter differ at P < 0.05.

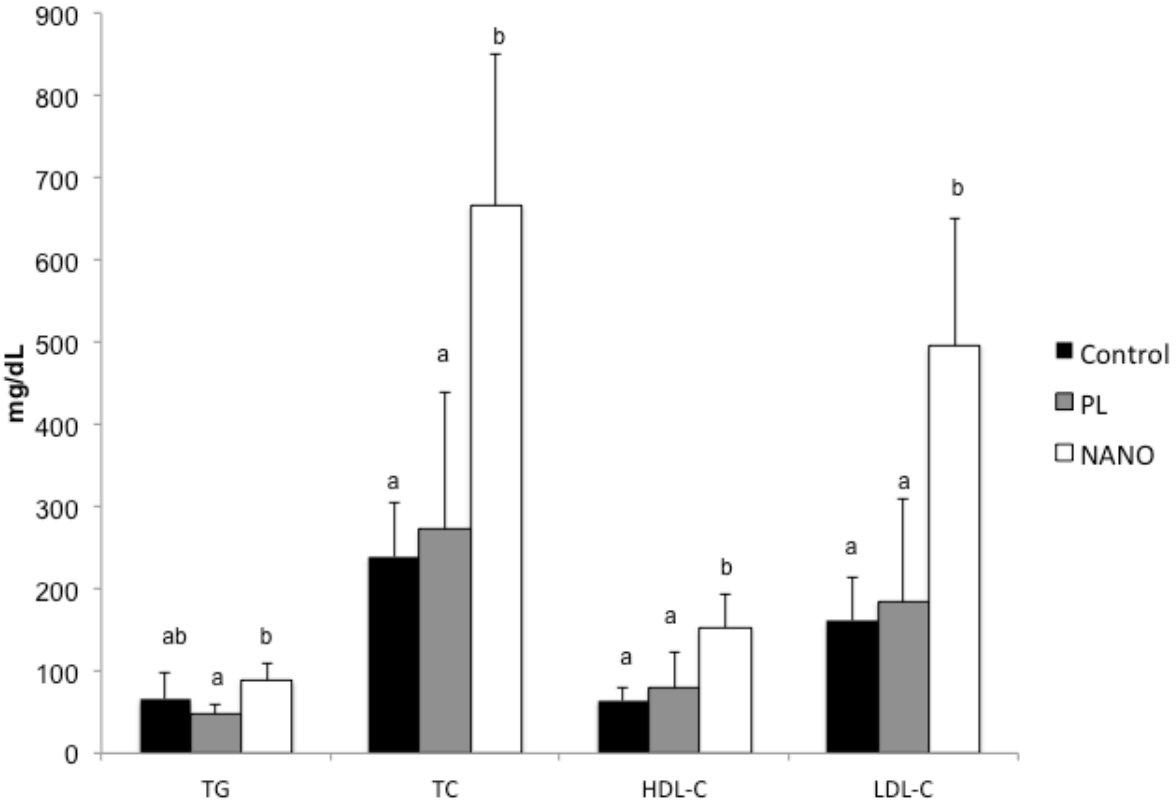


Figure 6.2 Pearson correlations between plasma lutein and LDL-C concentration (Panel A), HDL-C concentration (Panel B), number of LDL particles (Panel C), and number of HDL particles (Panel D) of guinea pigs fed the control, powdered lutein (PL) and lutein nanoemulsion (NANO) diets.

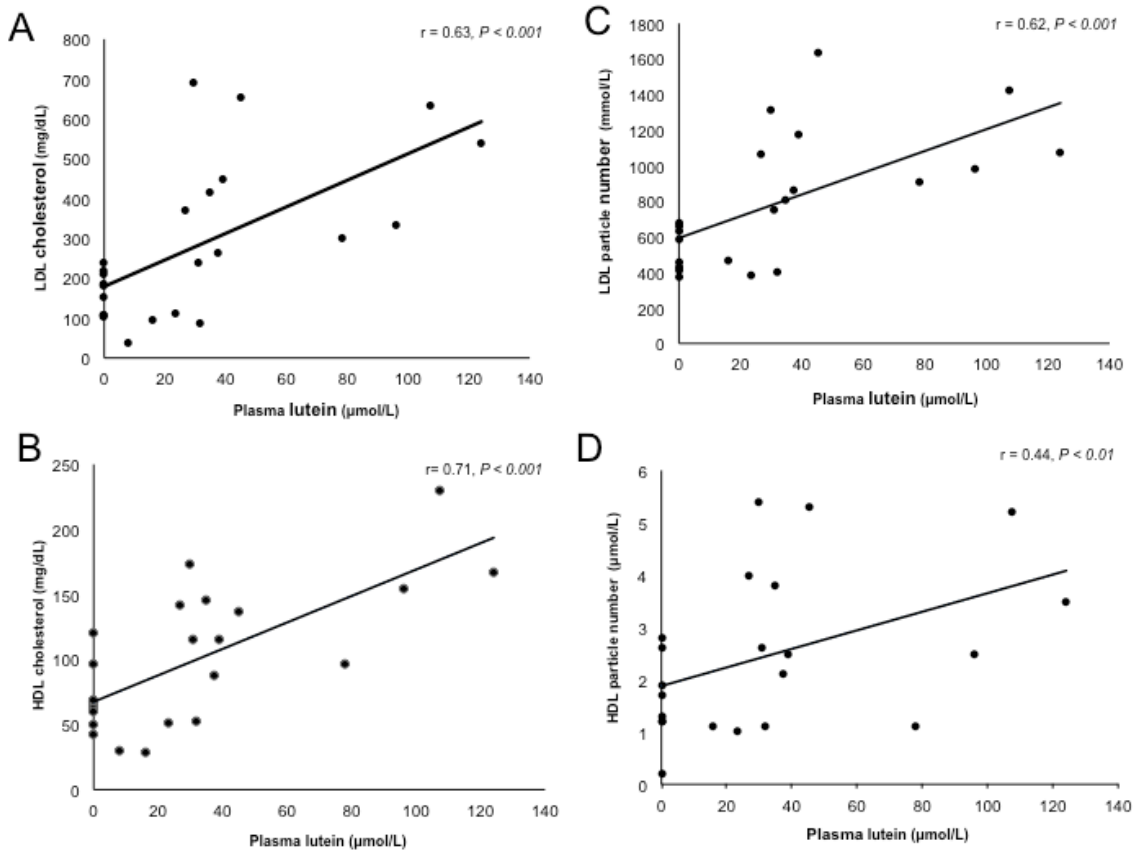
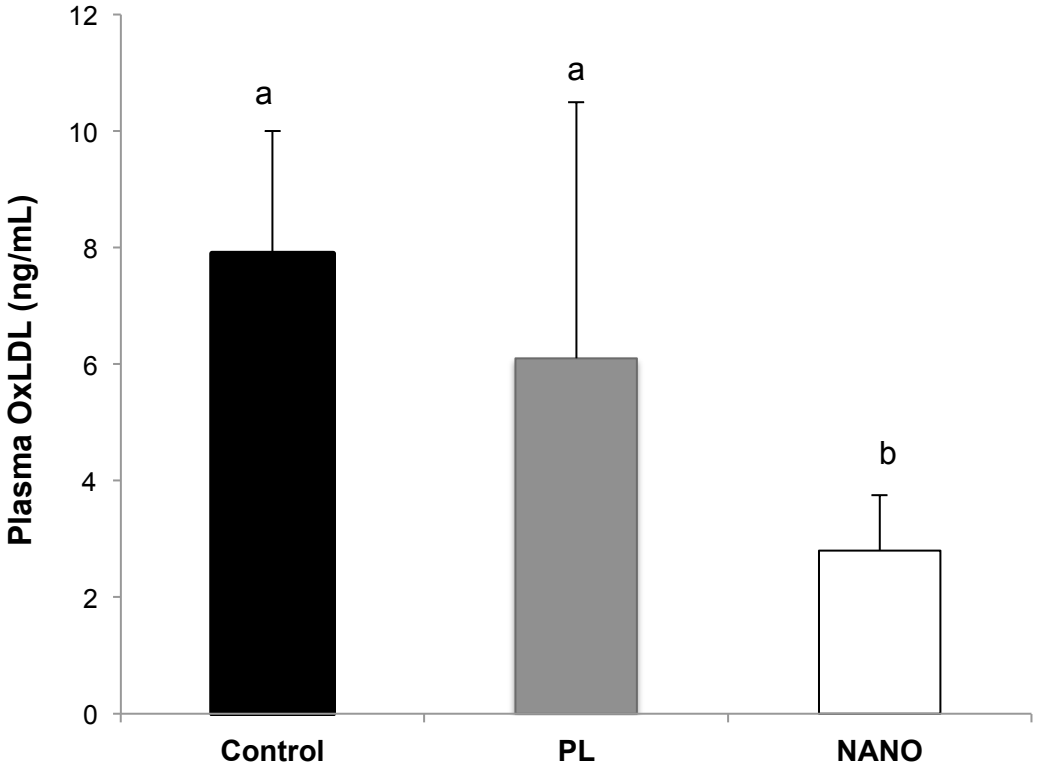


Figure 6.3 Concentrations of OxLDL in plasma of male guinea pigs fed a high cholesterol diet with no lutein (control), powdered lutein (PL) (3.5mg/d), or a nanoemulsion of lutein (NANO) (3.5mg/d) for 6 wks. Values are mean ± SD, n=8. Bars without a common letter differ at P < 0.05.



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Chapter 7

Conclusions and future directions

7.1 Conclusions

This project demonstrated that a nanoemulsion of lutein made with MCT oil and TPGS posses the characteristics of a stable and monodisperse preparation, which suggests that these ingredients are suitable for the successful nanoencapsulation of lipophilic nutraceuticals.

Most importantly, we confirmed our hypothesis that this nanoemulsion of lutein would be more bioavailable than the same dose of powdered lutein, as a higher concentration was found in both plasma and liver of the guinea pigs from the NANO group, when compared to both controls and PL, and in other tissues like adipose tissue and eyes, when compared to controls. These results suggest that nanotechnology is a good approach to overcome the challenges that bioactive components, in this specific case, lutein, have to exert their biological activity: low intake and poor bioavailability.

Moreover, the higher concentration of lutein were shown to be more effective in protecting the hepatic tissue against cholesterol-induced damage; as the NANO group showed lower liver weight, concentration of both TC and CE, ALT, steatosis score and DGAT2 mRNA expression. However powdered lutein also exhibited these protective effects, as there were no differences in hepatic oxidized OxLDL despite the different in tissue concentration.

However, an unexpected result was observed in the NANO group as these animals showed significantly higher concentrations of all lipoproteins, which means a shift

towards an atherogenic lipid profile. This negative result had a detrimental impact on the adipose tissue, as seen in a higher expression of pro inflammatory cytokines and FC. These results suggest that lutein's antioxidant and anti-inflammatory effects may not be sufficient in cases of severe hypercholesterolemia.

Overall, although the use of this particular nanoemulsion formulation may be beneficial in terms of increasing the concentrations of lutein in plasma and target tissues and exerting better hepato-protective bioactivity, it may be detrimental to the whole system, inducing an atherogenic lipid profile and aggravating the systemic inflammation caused by a cholesterol challenge.

7.2 Future directions

The importance of conducting this project lies in the attempt to surmount the knowledge gap that exists in using nanotechnology to improve bioavailability and efficacy of lutein. However, there are many questions that remained unanswered by the completion of the study.

The results presented in this dissertation showed that the nanoemulsion increased the bioavailability of lutein, but it was not elucidated how. The stability and behavior of the nano preparation in the digestive tract has yet to be tested with an *in vitro* digestion. Intestinal cell culture studies could also provide valuable information about the fate of the nanoemulsion particles in the intestine. In addition, the hypothesis that TPGS inhibits MDR1 in the enterocyte has yet to be confirmed.

An important limitation in this project was the lack of an empty vehicle control. A similar study should be conducted using a nanoemulsion without lutein to evaluate the individual or synergistic effects of the nanoemulsion ingredients, specifically TPGS and MCT oil in the systemic responses observed in this project. Another control that could be tested could be a group of guinea pigs receiving the same ingredients of the nano preparation but without being nanoemulsified. This will help us evaluate the differences in the effects that the ingredients have as a nano-delivery system versus the same materials in bulk.

In addition, the preparation used is not the only way to make nanoemulsions. Several surfactants and carrier oils can be used and tested in order to find the most efficient formulation that would increase the lutein concentration in target tissues without having the detrimental effects observed in this study.

In future studies, the effects of a nanoemulsion of lutein should be tested in several target tissues where lutein accumulates, such as the brain, lungs and spleen. In addition, the incorporation of lutein into the diet at the beginning of the study only gives us information about the potential of this carotenoid as a preventive agent, not as treatment. A study where lutein is added to the diet 6 weeks after the cholesterol challenge, to insure that damage is achieved could show the potential of lutein as a therapeutic, not only preventive, nutraceutical.

It could also be interesting to compare the efficacy of absorption of lutein between a nanoemulsion and egg yolks, because this dietary source is known to have the best natural composition (fat, free lutein, digestible matrix) to achieve maximum

bioaccesibility of this carotenoid. Finally, a study with humans is imperative to see if the effects observed in this study are reproducible and of clinical importance.

Although a promising alternative, the use of nanotechnology in oral formulations for nutraceuticals should be tested with other parameters of safety and efficacy in order to know if it is safe to move forward to perform clinical trials with oral nano formulations of lutein. So far, the use of this particular nanoemulsion should not be recommended for human oral supplementation and further animal or *in vitro* studies should be done before clinical trials can be conducted.