12-2017

# Effects of Vagotomy and Fenugreek on Hyperlipidemia and Insulin Resistance

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By

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A THESIS
Presented to the Faculty of
The Graduate College at the University of Nebraska
In Fulfillment of the Requirements
For the Degree of Master of Science

Major: Nutrition
Under Supervision of Professors
Qiaozhu Su and Dipak K. Santra

Lincoln, Nebraska

December 2017

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Rituraj Khound, M.S.

University of Nebraska, 2017

Advisor: Qiaozhu Su

Hyperlipidemia is the impairment of lipid metabolism marked by abnormally high levels of lipid in circulation. This has been implicated in a number of metabolic diseases including diabetes, cardiovascular diseases, and nonalcoholic fatty liver disease. Insulin resistance is the impairment of insulin action, which leads to several diseases such as obesity and type 2 diabetes. New clinical and therapeutic approaches are warranted for the prevention and treatment of hyperlipidemia and insulin resistance. In our study, we investigated the mechanism underlying the effect of complete disruption of the sub-diaphragmatic vagus nerve (vagotomy) on hyperlipidemia and insulin sensitivity. We observed that vagotomy significantly reduced the fasting and postprandial TG levels and VLDL synthesis. Vagotomy also resulted in elevated circulatory GLP-1 levels which possibly led to reductions in de novo fatty acid synthesis and VLDL production. This surgical procedure also found to ameliorate HFD-induced hyperlipidemia and hepatic steatosis. Significant improvement in insulin sensitivity was also observed in vagotomized mice. We also studied the effect of fenugreek seeds on hyperlipidemia using CREBH-null mouse model. We observed that consumption of fenugreek and treatment with trigonelline, a bioactive compound derived from fenugreek, ameliorated induced hyperlipidemia by upregulating transcription of Insig proteins, preventing VLDL synthesis and enhancing fatty acid oxidation. These findings rationalize the use of vagotomy as an effective clinical procedure to treat obesity and diabetes. The study also substantiates the prospect of using fenugreek as a potent nutraceutical in the treatment of metabolic diseases.

## Acknowledgements:

I would first like to thank my graduate advisor, Dr. Qiaozhu Su, for giving me the opportunity to pursue Master of Science in Nutrition at this esteemed university. I was able to learn the theoretical and technical fundamentals of Molecular Nutrition under her strict supervision. She was always there to provide her guidance, help and support throughout my study period. I am also thankful to my co-advisor, Dr. Dipak Santra, for his continual support and advices during my studies. He taught me how to diligently plan experiments and execute in an effective manner.

I was privileged to have Dr. Janos Zempleni and Dr. Soonkyu Chung as members of my advisory committee. I garnered immense knowledge in Nutrition by interacting with them in and out of their classes. I am grateful to both of them for their invaluable suggestions and support throughout my study period.

I would also like thank my fellow lab members, Jing Shen and Dr. Yongyan Song, for helping me learn the techniques when I started my project in the lab. I'm also thankful to Xiao Cheng, Fisayo Adenian and Neetu Sud for their help with my experiments. I offer my heartfelt thanks to David Giraud, Anjeza Erickson, Allison Rickey and Vernon Florke for their help. I'm immensely grateful to Lori Rausch, Diane Nelson, Diane Brown, and Amy Brown for all their help.

I am also thankful to Dr. Meenakshi Santra, Harleen Kaur, Sonal Sukreet, Suresh Varsani, and Ravi Mural, for their friendship, support and encouragement. They made my stay in Lincoln a memorable one.

I will always be indebted to my family for their unconditional love, constant support and motivation to do something meaningful in life. I'm also thankful to my fiancée for her love and her support, especially while I was preparing this thesis. Last but not the least, I would like to thank Almighty God for the strength to complete my masters at this esteemed institution.

## Table of Contents:

**List of Abbreviations:**

| | |
|---|---|
| apoB | apolipoprotein B |
| apoE | apolipoprotein E |
| TG | triglycerides |
| CHOL | cholesterol |
| VLDL | very low density lipoproteins |
| HDL | high density lipoproteins |
| LDL | low density lipoproteins |
| TRL | TG-rich lipoproteins |
| CREBH | cyclic AMP-responsive element-binding protein H |
| IRS-1 | insulin receptor substrate-1 |
| GLP-1 | glucagon-like peptide-1 |
| SCD-1 | stearoyl-CoA desaturase-1 |
| HFD | high fat diet |
| PA | palmitic acid |
| BSA | bovine serum albumin |

| | |
|---|---|
| DMEM | Dulbecco's modified Eagle's medium |
| FBS | fetal bovine serum |
| PCR | polymerase chain reaction |
| RT-qPCR | real time quantitative PCR |
| SDS-PAGE | sodium dodecyl sulfate polyacrylamide gel Electrophoresis |
| PVDF | polyvinylidene fluoride |
| Tris | tris (hydroxymethyl) aminomethane |
| TBST | Tris-buffered saline with tween |
| FPLC | fast protein liquid chromatography |
| NaCl | sodium chloride |
| $CaCl_2$ | calcium chloride |
| DTPA | diethylenetriaminepentaacetic acid |
| $NaN_3$ | sodium azide |
| SEM | standard error of the mean |
| OGTT | oral glucose tolerance test |
| Vag | vagotomized |

| | |
|---|---|
| Veh | vehicle |
| KO | knockout |
| Fenu | fenugreek |
| Trigo | trigonelline |
| CNS | central nervous system |

# Chapter One: General Introduction

## 1.1 Hyperlipidemia

Hyperlipidemia, an impairment of lipid metabolism marked by an abnormal elevation of lipid in blood plasma, is rapidly increasing around the world [1]. It is linked to the pathogenesis of myriads of chronic metabolic diseases such as nonalcoholic fatty liver disease (NAFLD), insulin resistance and atherosclerosis [2]. Hyperlipidemia involves elevated serum total cholesterol (TC), triglycerides (TG), and low –density lipoprotein cholesterol (LDL-C) and decreased levels of high-density lipoprotein cholesterol (HDL-C) [3]. Increased LDL-C in circulation is considered a high risk factor of atherosclerosis (AS) and coronary heart disease (CHD). Elevated levels of circulatory triglycerides (TG) and free fatty acids (FFA) also contribute to AS and CHD. High prevalence of hyperlipidemia is intrinsically linked to increased risks of cardiovascular diseases (CVD) [1]. It is imperative to devise strategies to modulate the dysregulation of lipid metabolism and reduce the levels of blood TC, TG and LDL-C in order to ameliorate CVD. Lipid-lowering drugs like statins, fibrates, and nicotinic acid, are normally prescribed for treating hyperlipidemia. However, these drugs have limitations due to side effects and poor tolerability in some patients [3].

CREBH, c-AMP responsive-element- binding protein H, is associated with hyperlipidemia. The CREBH, encoded by *CREB3L3* gene in human, is an ER-bound transcription factor expressed extensively and exclusively in the liver and small intestine [4]. It belongs to the CREB/ATF family, and consists of a basic leucine zipper (bZIP) domain and a transmembrane domain [5, 6]. Proteolytic cleavage between the bZIP

domain and the transmembrane domain releases the ER membrane-bound CREBH protein which then translocate to the nucleus for transcriptional regulation of target genes [7, 8]. It was later reported that ER stress is responsible for expression and cleavage of CREBH protein via response intramembrane proteolysis (RIP) in the hepatocytes [9]. Besides, bacterial endotoxin lipopolysaccharides and proinflammatory cytokines such as TNF-α, IL-6 and IL1β can also lead to expression and activation of CREBH. Nutritional cues such as fasting and fatty acid can induce CREBH expression and refeeding suppresses the expression [2, 4, 6]. These transcription factors have been found to be involved in glucose and lipid metabolism [5, 10, 11].

Disruption of the *CRE3L3* gene, which encodes CREBH, expression leads to disorders in lipid metabolism. Nonsynonymous mutations in *CREB3L3* gene was reported to develop non-functional CREBH, which led to extreme hypertriglyceridemia in mice suggesting a significant role of CREBH in triglyceride metabolism [4]. In another study, atherogenic high fat diet fed CREBH-null mice showed hepatic lipid accumulation and increased plasma TG levels indicating a crucial role of CREBH in maintaining hepatic lipid homeostasis [5]. Other studies also emphasize the role of CREBH in the regulation of plasma TG levels which suggest CREBH as an antiatherogenic transcription factor [10, 11]. Studies have also established a link between CREBH expression, metabolic inflammation, VLDL metabolism, and hyperlipidemia [12]. Therefore, reduced levels of CREBH is one of the major factors associated with hyperlipidemia.

**1.2 Insulin Resistance:**

Insulin is an endocrine hormone secreted by pancreatic β-cells in response to nutrients uptake, primarily glucose. The secretion of insulin, however, is also augmented by free fatty acids and amino acids in circulation. [13]. Insulin is the primary hormone of energy homeostasis as it stimulates influx and utilization of glucose in the muscle, glycogen synthesis in the liver and muscle, fat storage in the adipocytes, and suppression of hepatic glucose uptake [14-17]. Insulin action involves an intricate set of signals which enables the body to balance nutrient availability and requirements [17].

Insulin resistance (IR) refers to impaired insulin action manifested by a decreased response of the peripheral tissues to a certain concentration of insulin [18, 19]. This pathological condition leads to several disease states, including hypertension, glucose intolerance, obesity, dyslipidemia, type 2 diabetes, low HDL cholesterol, and accelerated atherosclerosis [18, 20]. IR may develop in different organs like the liver, muscle, kidney, adipose tissue and pancreas [21]. The lack of insulin action leads to increased hepatic glucose production, decreased glycogen synthesis, and reduced glucose uptake by muscle, resulting in increased levels of glucose in circulation which is the well-recognized feature of IR [14, 21].

The exact reason for IR still remains unclear despite different postulations aimed to elucidate the underlying mechanism [22]. Nevertheless, some of the contributing factors include impaired insulin receptor substrate (IRS) and glucose transporter protein (GLUT), β-cell dysfunction, phosphorylation of intracellular glucose, oxidative stress, free fatty acids and adipokines [21, 23]. Free fatty acids (FFA) have been widely

considered as a critical factor in development of IR [17, 23, 24]. FFA may induce IR by impairing glucose transporter (GLUT), a protein that mediated translocation of glucose into muscle [25]. Proinflammatory adipokines, such as TNFα and IL-6, released by obesity-induced hypertrophic adipocytes may also induce insulin resistance by turning the insulin signaling down via serine phosphorylation of IRS-1 [25-28]. In the absence of insulin regulation, the flux of FFA from adipocytes to other tissues such as hepatocytes, pancreas and muscle tissue is augmented via increased lipolysis. This further deteriorates insulin activity and leads to other adverse effects such as lipotoxicity [28].

**1.3 Vagus Nerve Signaling and GLP-1 Secretion:**

The vagus nerve is the principal nerve of the parasympathetic nervous system, one of the three divisions of the autonomic nervous system [29]. The afferent and efferent signaling of the vagus nerve plays an important role in the regulation of feeding behavior and metabolic homeostasis [29]. Abdominal vagal efferent activity regulates secretion of gastric and pancreatic digestive juices and anabolic hormones [30]. Vagotomy is the surgical removal of the vagus nerve. Sub-diaphragmatic vagotomy reportedly reduced obesity and improved fasting insulinemia, glucose tolerance and insulin sensitivity in monosodium glutamate-treated (MSG) mice [31, 32]. However, the underlying mechanisms are still not elucidated.

Glucagon-like peptide-1 (GLP-1) is an incretin hormone secreted in the intestine in response to food intake and hormone regulation [33, 34]. Among all other nutrients, glucose and monounsaturated fatty acids are the primary stimulus of GLP-1 secretion [35]. GLP-1 is synthesized by the enteroendocrine L cells of ileum and proximal colon

[36, 37]. GLP-1 is also synthesized in the brain as a neuropeptide where it contributes to central regulation of metabolic and cardiovascular functions [38, 39]. These peptides are implicated in glucose-dependent insulin secretion, glucagon inhibition, gastric emptying, and enhanced satiety [34, 36, 37, 39, 40, 41, 42, 43]. Besides, GLP-1 modulates β-cell apoptosis, activates hepatoportal glucose sensors, and promotes β-cell growth and preservation, and peripheral insulin sensitivity [44- 47]. Moreover, GLP-1 action attenuates postprandial secretion of large, triglyceride containing lipoproteins in plasma [36, 42]. Native GLP-1 has also been found to prevent development of atherosclerotic lesions [48, 49]. Recent studies reported the potential role of these incretin peptides in recruitment of muscle vasculature, augmented delivery of insulin to muscle, and improvement of insulin action in insulin-resistant states [50].

Stearoyl-CoA desaturase (SCD) participates in the de novo synthesis of monounsaturated fats from saturated fatty acids. One of the major products of this conversion is oleate which is the major monounsaturated fatty acid of phospholipids of the bi-layered membrane, triglycerides, cholesterol esters, wax esters, and diacylglycerol. Two SCD isoforms viz., SCD-1 and SCD-2 have been identified in humans. Adipose and liver tissues express highest levels of SCD-1 expression, while SCD-2 is primarily expressed in brain and pancreatic tissues [51]. Although no protection from obesity was observed, simultaneous deletion of SCD-1 in liver and adipose tissues reportedly lowered TG levels in the liver and improved glucose tolerance [52]. It has also been established that there is a relation between SCD-1 mRNA expression and 16:1/16:0 activity indices in plasma VLDL-TG [53]. Keeping these observations in view, SCD-1 mRNA expression can possibly be used as a reliable tool to assess alterations in lipid metabolism in the liver.

**1.4 Fenugreek:**

Fenugreek (*Trigonella foenum-graecum* L.) is an annual crop that belongs to Leguminosae family. Fenugreek has mostly been used as a spice in various parts of the world to improve the sensory quality of food [54]. This crop is widely cultivated in most regions of the world, particularly in India, Pakistan, China, Egypt, Ethiopia, Morocco and England [55-57].

Fenugreek seeds are used in India and other Asian countries as a spice in food preparations owing to their strong aroma and flavor [58, 59]. The leaves are also consumed in India as a green, leafy vegetable as they are a rich source of calcium, iron, β-carotene and other vitamin content [60]. Besides, the extract of fenugreek is used as a flavoring agent to make cheese in Switzerland, artificial maple syrup and bitter-run in Germany, as substitute to coffee in Africa, as fortification to make bread in Egypt, anti-diabetic herb in Israel, as insect and pest repellent in grain storage in France [56,60]. Fenugreek is used as herbal medicines in many regions of the world [60]. They have long been used in traditional Ayurvedic, Unani and Chinese medicinal systems for treatment of numerous human health problems like diabetes, indigestion, epilepsy, paralysis, gout, chronic cough, and as a general oral medicine to improve metabolism [57, 59, 61].

Several important chemical compounds of medicinal value have been identified in fenugreek leaves and seeds. Fenugreek has been recognized among common nutraceutical products because of the presence of three important chemical constituents. The seeds are a good source of important steroidal sapogenin like diosgenin, the polysaccharide galactomannans, and isoleucine. Fenugreek is known to have several

pharmacological properties such as hypoglycemic, hypocholesteorolemic, antioxidant, anti-inflammatory, and anti-ulcerogenic [55, 59, 62]. The amino acids histidine and hydroxyisoleucine in fenugreek reportedly plays a key role in insulin sensitivity [63]. Optimal consumption of fenugreek has been reported to reduce triglycerides and cholesterol levels in plasma [64]. Study with ethyl acetate extract of fenugreek seeds showed lowered LDL-triglycerides and cholesterol and increased HDL-cholesterol [65]. The findings in these reports indicate that bioactive compounds in fenugreek seed are possibly involved in lipid metabolism.

Trigonelline is a natural plant alkaloid which was first isolated from fenugreek seeds [66, 67]. This bioactive compound is known to possess hypoglycemic and hypolipidemic properties [67]. Trigonelline is highly regarded as an anti-diabetic owing to its ability to improve insulin sensitivity by enhancing insulin signaling [68] and by ameliorating ER stress and oxidative stress-mediated pancreatic cell damage [67], increased insulin secretion, inhibit adipogenesis, upregulate antioxidant enzyme activity, decrease lipid peroxidation, and prevent postprandial hyperglycemia [66-70]. Trigonelline has also been reported to possess inhibitory effects on cancer and non-alcohoic fatty liver diseases (NAFLD) [71, 72].

### 1.5 Working Hypothesis

We hypothesize that vagotomy leads to alteration in the levels of glucagon-like peptide-1 (GLP-1), which in turn inhibits hepatic very low density lipoprotein (VLDL) assembly and secretion, leading to the amelioration of hyperlipidemia and insulin resistance induced by high-fat-diet (HFD). Consumption of fenugreek seeds also modulates hepatic

VLDL secretion and improves insulin sensitivity by modulating the expression of genes involved in fatty acid metabolism and β-oxidation, and alleviating ER stress-induced inflammation.

**1.6 Study Objectives:**

**Aim-1:** The goal of this study is to assess the role of GLP-1 in modulation of lipoprotein metabolism in vagotomy mice. We will first investigate the role of GLP-1 in modulation of hepatic lipid metabolism. We will further study any possible impact of vagotomy on insulin sensitivity and glucose tolerance.

**Aim-2:** In aim-2, we will elucidate the role of fenugreek seeds in regulation of *de novo* lipid synthesis and further investigate the effect of the bioactive compound trigonelline on hyperlipidemia.

## Chapter Two: Materials and Methods

### 2.1 Animals and Animal Experiments:

For in vivo studies, 12-week old sham and vagotomized (sub-diaphragmatic) mice were purchased from Jackson Laboratory (Bar Harbor, Maine, USA). Animals were housed under alternating 12 h light and dark conditions with unrestricted access to food and water. All the mice were placed on a chow diet (Dyets Inc., USA). Animals were used for specific experiments following a week of acclimatization. Baseline blood was collected from the animals after a 12 h fast. In order to use for high fat feeding studies, animals were fed a regular chow diet ad libitum or a high fat diet (HFD) (11.5% coconut oil, 11.5% corn oil, 5% fructose, and 0.5% cholesterol) (Purina Rodent Chow no. 5001, #C11953; Research Diets, New Brunswick, NJ, USA) for 22 weeks. Body weight, food intake, and plasma lipids were monitored throughout the feeding period. After 22 weeks of HFD feeding, tissues were collected from the animals following a 5 h fasting and subsequent anesthetization using isoflurane (3% mixed with oxygen). The tissues were snap frozen in liquid nitrogen and stored at -80% for future experiments.

For studies with fenugreek, 10-14 week old CREBH knockout mice, with deletion of exons 4-7 of the CrebH gene, were used [5, 9]. The animals were housed with alternating 12-hour light and dark cycles with free access to food and water and placed on a standard chow diet (Envigo, Madison, Wisconsin. #2016). CREBH-null mice were randomly divided into two groups (n=10/group) and given either a standard chow with vehicle (Nutriacal gel, Vetoquinol, Princeville, Quebec) or chow containing 2% of fenugreek whole seed for 7 weeks. The fenugreek seeds (cultivar Tristar) were provided by Dr. Dipak Santra (Panhandle Research and Extension Center, University of Nebraska-Lincoln). Fenugreek

was grown in Research Farm of Panhandle Research and Extension Center at Scottsbluff, NE (41.890746, -103.676169) under irrigation in 2014 growing season. After completion of the 7 weeks feeding procedure, the mice were euthanized by isoflurane (3% mixed with oxygen) following 12 h of fasting. Blood samples from the heart were collected in lithium heparin coated capillary tubes (BD Vacutainer Labware Medical, USA). Plasma from the blood samples were separated by spinning at 6000 rpm at 4$^{o}$C for 10 min, and were stored at -80$^{o}$C for future analyses. The liver tissues were snap frozen in liquid nitrogen and stored at -80$^{o}$C for further analyses.

**2.2 Cell Culture Treatments:**

For in vitro studies, rat hepatoma cell lines, McA-RH7777 (McA) (ATCC) cells were used, which were maintained in DMEM containing 10% FBS, 4.5 g/L glucose and 0.5% penicillin/streptomycin at 37°C, 5% $CO_2$. The cells were cultured in flasks with media changes every alternate day and regularly passaged every 3-4 days. 3 x $10^6$ cells were seeded onto 6-well plates 24 h prior to treatment. Palmitic acid (PA) was prepared as previously described [73]. The cells were subsequently treated with exendin-4 (Exenatide H-8730, Bachem, Bubendorff) (100 nm), palmitic acid (PA) (50 μM) or vehicle (0.5% BSA) for 48 h in DMEM devoid of FBS. Primary hepatocytes, isolated from 12-week old C57/B6 male mice, were seeded (1.3 x $10^6$) onto 6-well plates and cultured in William's R medium containing 5% FBS, 0.5% P/S, 1% L-Glutamine. Cells were then treated with exendin-4 (100 nM), PA or vehicle (0.5% BSA) for 48 h in William's E medium without FBS. Media was collected for measuring secreted TG and CHOL.

For trigonelline treatment, 3 x $10^5$ cells were seeded onto 6-well plates and maintained in complete DMEM (4.5g/L glucose). When the cells reached 60-70% confluence, they were treated with 100 μM of trigonelline (Sigma-Aldrich, St. Louis, MO) for 24 h followed by a 12 h fast. After the 12 h fasting, the cells were then treated with palmitic acids (PA) in the presence of mock ($H_2O$) or trigonelline (100μM) for additional 6 h. The media were collected for lipid extraction and estimation as described in a previous publication. [74]

**2.3 RNA Expression Studies:**

Total RNA was extracted from the cryo-preserved livers of CREBH-null mice fed with chow diet along with 2% fenugreek seeds, as well as vagotomized and sham mice. The mRNAs from tissues were extracted with TRIzol reagent (Life Technologies, Grand Island, NY) according to the protocol provided by the manufacturer. Integrity of the isolated mRNAs was confirmed using a NanoDrop 2000 (Wilmington, DE). The mRNAs were reversed with a high capacity cDNA reverse transcription kit with RNase inhibitor (Life Technologies). The expression levels of peroxisome proliferator-activated receptor α (PPARα), carnitine palmitoyltransferase (CPT-1α), peroxisomal acyl-coenzyme A oxidase 1 (Acox-1), Insulin-induced gene 1 (Insig1a), insulin-induced gene 2b (Insig2b), medium-chain acyl-CoA dehydrogenase (MCAD), fatty acid synthase (FASN), acetyl CoA carboxylase (ACC), sterol regulatory element binding protein 1c and 2 (SREBP-1c and SREBP-2), and SCD-1 mRNA were measured by quantitative real-time PCR (qRT-PCR) using SYBR Green PCR Master Mix (Applied Biosystems, Life Technologies).

18S rRNA was used as an internal reference to calculate relative quantities of mRNA from threshold cycle ($C_T$) values with the comparative $C_T$ method.

**2.4 Lipid Extraction and Lipid Content Measurements:**

To extract liver lipid, approximately 300 mg of liver tissue was added to 20 volumes of 2:1 chloroform: methanol mixture and incubated at room temperature for 24 h. After the incubation, 0.2 volumes of 0.9% NaCl was added to the solvent mixture. The samples were centrifuged at 2000 rpm for 3 min following a thorough vortex. The upper aqueous layer was discarded and the volatile solvent layer was allowed to air dry. The dried lipid was resuspended in 1 ml of 100% ethanol. Lipid from cultured cells were extracted by 3:2 hexane: isopropanol, dried and resuspened in 100% ethanol [46]. In order to study the alterations in lipid in circulation, 2 μl of plasma was used to measure the lipid contents. The levels of TG and CHOL were measured using an enzymatic/GPO lipid assay kit (Pointe Scientific, Canton, MI). The assay was done in accordance with the protocol provided by the manufacturer.

**2.5 Immunoblot Analysis:**

Proteins were extracted from the treated cells using 1x RIPA lysis buffer (150 mM sodium chloride, 10 mM Tris pH 7.4, 1mM EDTA, 1% Nonidet P-40, and protease inhibitor cocktail tablet). Protein samples were prepared with 4x SDS loading buffer (200 mM Tris-HCL, pH 6.8, 8% SDS and 0.002% bromophenol blue) and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After separation, proteins were transferred overnight onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA) under a voltage of 40V at 4$^{o}$C in a wet transfer

apparatus (Bio-Rad Laboratories Ltd., Mississauga, ON). The membranes were blocked in 5% milk in 1X TBST for at least 2 hours on a shaker at room temperature. The membranes are then washed with 1x TBST 5 times for 5 minutes and were subsequently incubated with primary antibodies overnight at $4^{o}$C. The following antibodies were used for immunoblotting: anti-apoB, anti-apoE, and anti-albumin (Nittobo America Inc., Murrieta, CA); anti-β-actin (Sigma Aldrich, St. Louis, MO); anti-phospho-Akt, anti-SCD-1, and anti-insulin receptor β (Cell Signaling, Danvers, MA); anti-SREBP-1c and anti-SREBP-2 (Novus, Littleton, CO). The final concentration of all the antibodies used was 0.1- 1 µg/ml). The membranes were washed 5 times for 5 minutes with 1X TBST the following day and incubated with specific horseradish peroxidase-conjugated IgG secondary antibodies. Anti-mouse, anti-rabbit (GE Healthcare, Buckinghamshire, UK), or anti-goat (Santa Cruz, Dallas, TX) IgG secondary antibodies were used in the study. The membranes were again washed in 1x TBST 5 times for 5 minutes and treated with Luminol enhancer solution for 2 minutes for detection by enhanced chemiluminescence (ECL) (Pierce, Rockford, IL). The proteins were visualized in FluorChem E system (Protein Simple, San Jose, CA). Protein bands were quantified using Image J (NIH).

**2.6 Oral Glucose Tolerance Test (OGTT):**

The mice were fasted for 12 h overnight prior to oral glucose tolerance test. The baseline blood glucose concentration was first measured via tail vein bleeding. The mice were then given glucose by oral administration appropriate to their body weight (2g/kg). Blood glucose concentrations were measured at 15, 30, 60 and 90 min after glucose administration using a handheld glucometer (Contour/Next, Beyer).

**2.7 Lipoprotein Characterization:**

The lipoprotein subclasses in the plasma were separated using gel filtration fast protein liquid chromatography (FPLC). 150 μL plasma samples were injected onto a Superose 6 10/200 GL column (Amersham Pharmacia Biotechnology, Piscataway, NJ). The flow rate of the whole process was 0.5 ml/min. Specific eluent was used to elute the different fractions from the column. The TG and CHOL contents in each of the fractions were subsequently measured. The recipe of the eluent is: 10 mM Tris, 150 mM NaCl, 2 mM $CaCl_2$, 100 μM DTPA, 0.02% $NaN_3$, pH 7.4.

**2.8 Plasma GLP-1 Measurement**

GLP-1 in plasma was analyzed the GLP-1 (active 7-36) ELISA kit (Cat # 43-GP1HU-E01, Alpco Diagnostics, Salem, NH) according to the manufacturer's instructions.

**2.9 Statistical Analysis:**

Data acquired in the experiments were evaluated using one-way ANOVA (GraphPad Prism 5, La Jolla, CA, USA). The two-tailed Student's t-test was used for statistical analyses of two-group comparisons. All results are presented as mean ± SEM. Asterisks (* or **) indicate statistically significant differences of $P < 0.05$ or $P < 0.01$, respectively, compared to controls.

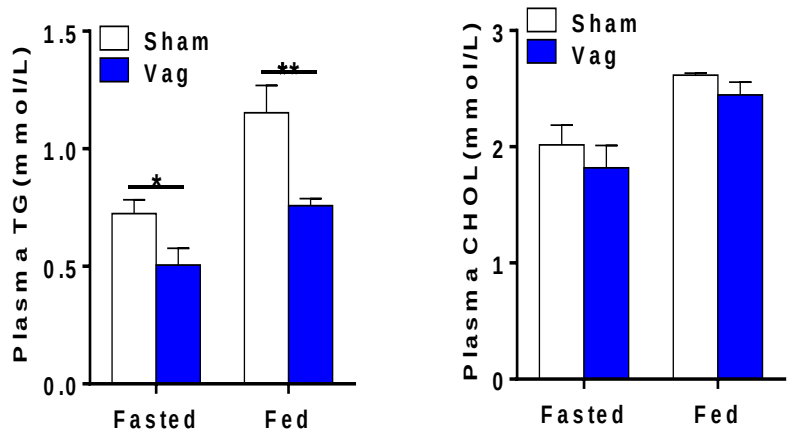
# Chapter Three: Results

## 3.1 Vagotomy

### 3.1.1 Sub-Diaphragmatic Vagotomy Reduces Circulatory TG and Protects Mice from Hyperlipidemia.

To determine the effect of sub-diaphragmatic vagotomy on lipid metabomism, we first investigated fasted and fed (ad libitum) circulating lipid levels. There was a significant reduction in circulatory TG levels at both fasted and fed states in vagotomized mice as compared to sham mice. There were no significant changes in plasma CHOL levels (Fig. 1A). FPLC was subsequently employed to determine the sub-class of lipoproteins which was affected by vagotomy. Lipid measurements revealed that there was a significant reduction (three fold) in VLDL-TG content in vagotomized mice while no changes in LDL or HDL associated TG was observed. CHOL content in all fractions were comparable in both types of mice (Fig. 1B). This suggests that vagotomy primarily regulates VLDL-TG metabolism.

**A**



**B**

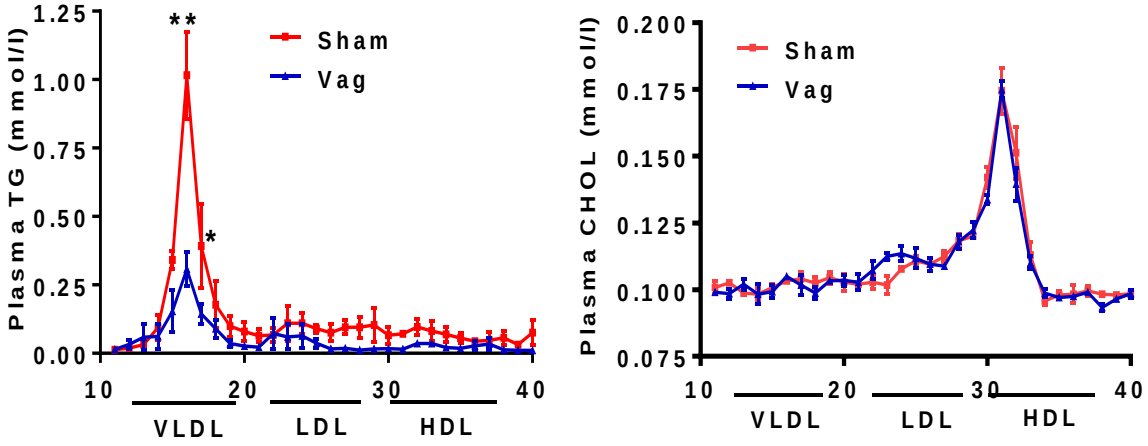



**Fig. 1:** Disruption of sub-diaphragmatic vagal signaling inhibits circulatory TG. Both sham and vagotomized mice were fed chow diet and blood was drawn from both the groups at the age of 12 weeks in fasted and fed states, and used for the following analyses (A) TG and CHOL contents in the plasma of sham and sub-diaphragmatic vagotomized (Vag) mice. (B) FPLC plasma fractions showing TG and CHOL contents in the sham and vagotomized mice. Results are shown as mean± SEM, n=4-7/group. *P<0.05 and **P<0.01 versus control.

In order to study the effect of vagotomy on postprandial hyperlipidemia, the mice were subjected to acute oral fat load of olive oil (200µl) and plasma TG and CHOL were measured. Plasma TG was significantly reduced in vagotomized mice whereas no changes were observed in plasma CHOL levels (Fig. 2A). Immunoblotting analyses of plasma proteins showed significant reduction in the levels of apoB48, the structural

protein component of intestinal chylomicrons, which suggests that vagotomy inhibits the secretion of intestinal TG-rich lipoproteins (TRL) resulting in decreased postprandial lipemia (Fig. 2B). These findings indicate that vagotomy decrease hyperlipidemia by regulating TG-rich lipoprotein metabolism.

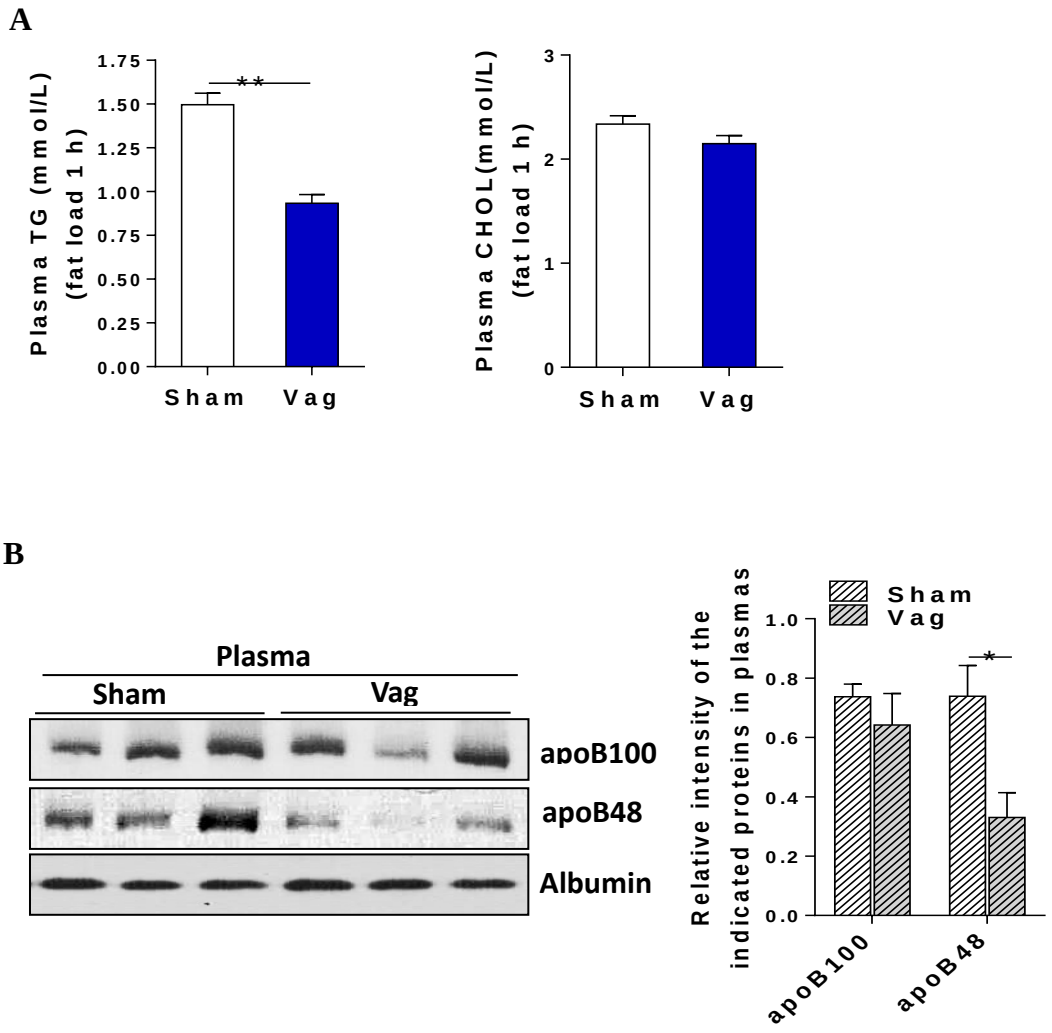
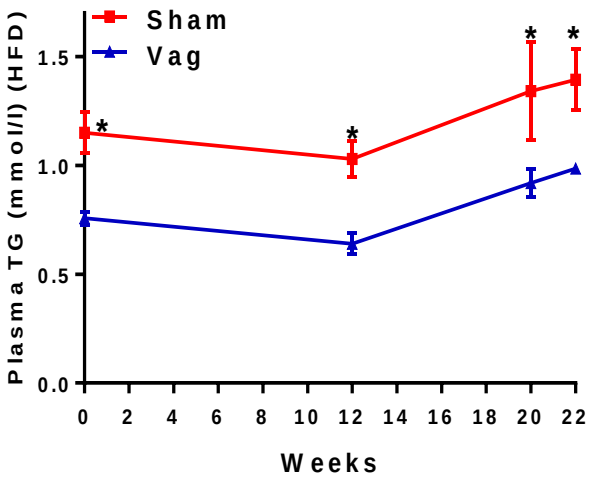


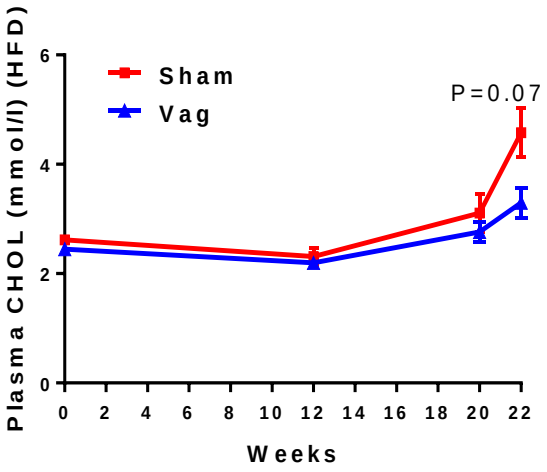
**Fig. 2:** Disruption of sub-diaphragmatic vagal signaling protects mice from HFD-induced hyperlipidemia. Both sham and vagotomized mice were subjected to acute oral fat load of olive oil (200µl) following 12 hours of fasting and the plasma was used for the following analyses (A) plasma TG and CHOL contents in sham and sub-diaphragmatic vagotomized mice. (B) Immunoblotting analysis of plasma apoB100, apoB48, and albumin at 1 h after a fat load (200 µl olive oil). Results are shown as mean± SEM, n=4-7/group. *P<0.05 and **P<0.01 versus control.

### 3.1.2 Sub-Diaphragmatic Vagotomy Prevents HFD-Induced Hyperlipidemia and Hepatic Steatosis.

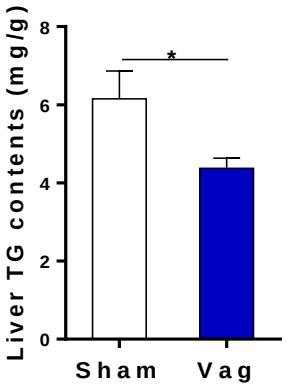
Two cohorts of sham and vagotomized mice were fed a HFD diet for 22 weeks to study the impact of vagotomy on HFD induced obesity and hyperlipidemia. Plasma TG at week 0, 12, 20, and 22 was significantly decreased in vagotomized mice compared to sham mice. There were no changes in plasma CHOL among both the mice types (Fig. 3A). In order to assess the impact of vagotomy on HFD-induced hepatic steatosis, the lipid content in the liver was measured in both sham and vagotomized mice. Hepatic TG was significantly reduced in vagotomized mice compared to sham controls, with no differences in CHOL (Fig. 3B). All these results suggest vagotomoy can prevent hyperlipidemia and hepatic steatosis induced by HFD.

**A**


Sham
Vag
Plasma TG (mmol/l) (HFD)
1.5
1.0
0.5
0.0
0 2 4 6 8 10 12 14 16 18 20 22
Weeks
*
*
*
*


Sham
Vag
P=0.07
Plasma CHOL (mmol/l) (HFD)
6
4
2
0
0 2 4 6 8 10 12 14 16 18 20 22
Weeks

**B**


Liver TG contents (mg/g)
8
6
4
2
0
Sham
Vag
*

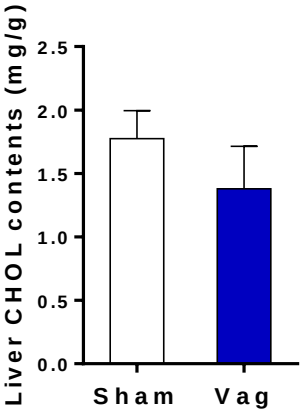
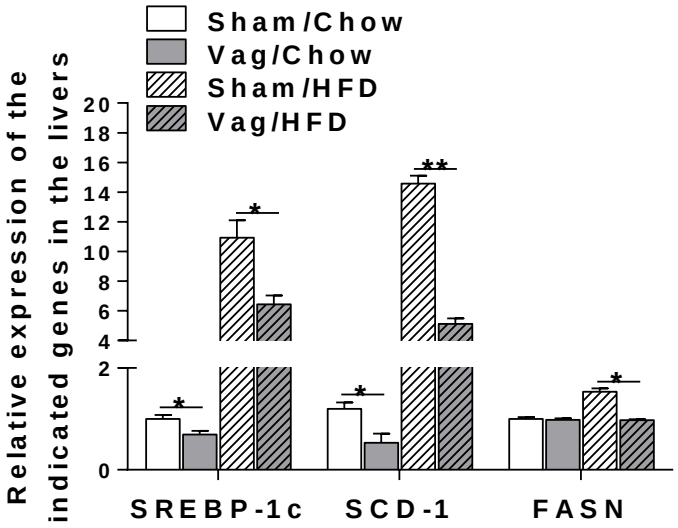

Liver CHOL contents (mg/g)
2.5
2.0
1.5
1.0
0.5
0.0
Sham
Vag

**Fig. 3:** Sub-diaphragmatic vagotomy prevents HFD-induced hyperlipidemia and hepatic steatosis. Sham and vagotomized mice were fed a HFD for 22 weeks. Blood and liver were collected for the following analyses. (A) Plasma TG and CHOL in sham and vagotomized mice at 0, 12, 20 and 22 after HFD (B) Liver TG and CHOL contents from sham and vagotomized mice after 22 weeks of high fat diet feeding. Results are shown as mean± SEM, n=4-7/group. *P<0.05 and **P<0.01 versus

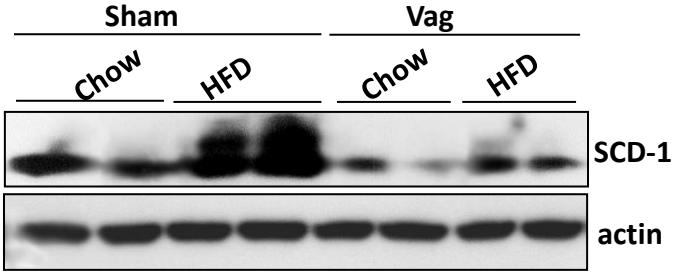
### 3.1.3 Vagtotomy Inhibits HFD-Diet Induced Hepatic Steatosis via Downregulation of Lipogenic Genes.

Improvement in lipid profile in HFD-fed vagotomized mice encouraged us to investigate the changes in hepatic lipid metabolism. We investigated two key genes involved in hepatic de novo synthesis viz., SREBP-1, SCD-1 and FASN. The mRNA levels of SREBP-1was found to be significantly lower in vagotomized mice compared to sham mice (Figure 4A). Furthermore, both mRNA and protein levels of SCD-1 gene were also considerably reduced in the liver of chow and HFD-fed vagotomized mice (Fig. 4A and 4B). Inhibition of SCD-1 possibly reduced OA production resulting in reduced availability of lipid for VLDL synthesis. Moreover, HFD could not significantly induce mRNA expression of FASN, a key lipogenic gene associated with free fatty acid biosynthesis, in vagotomized mice post HFD feeding (Fig. 4A). These observations are supported by reduced TG levels in the liver as mentioned in the previous section. These findings indicate that vagotomy significantly impairs de novo hepatic lipogeneis resulting in reduction in hepatic steatosis.

A


Sham/Chow
Vag/Chow
Sham/HFD
Vag/HFD
Relative expression of the indicated genes in the livers
20
18
16
14
12
10
8
6
4
2
0
*
*
**
*
SREBP-1c
SCD-1
FASN

B


Sham
Vag
Chow
HFD
Chow
HFD
SCD-1
actin

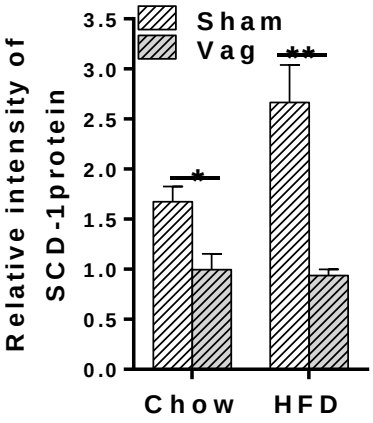

Sham
Vag
Relative intensity of SCD-1protein
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
**
Chow
HFD

**Fig. 4:** Disruption of sub-diaphragmatic vagus nerve prevents HFD-induced hepatic steatosis via down regulation of lipogenic genes. Sham and vagotomized mice were fed a HFD for 22 weeks. The liver was collected and used for the following analyses. (A) Relative mRNA expression of SCD-1 and FASN in the livers of sham and vagotomized mice after 22 days of high fat diet or control chow feeding. (B) Immunoblotting analysis of hepatic expression of SCD-1 protein in sham and vagotomized mice. Results are shown as mean± SEM, n=4-7/group. *P<0.05 and **P<0.01 versus control.

### 3.1.4 GLP-1 Secretion is Elevated Which Inhibits Hepatic de novo Lipogenesis.

GLP-1 has been implicated in dyslipidemia improvement via reduced lipoprotein production. We investigated the circulatory levels of GLP-1 to determine if it mediates the inhibitory effect of vagotomy on hepatic lipid synthesis. GLP-1 levels in the plasma were significantly increased in vagotomized mice in both fasted and fed states (Fig. 5A). The elevation was found to be more profound in the fed state. To study the changes in GLP-1 in postprandial state, the mice were administered a fat load comprising 200 µl olive oil. The fat load elevated the levels of GLP-1 in vagotomized mice 4 and 3 folds compared to fasted and fed states, respectively (Fig. 5B). This suggests that GLP-1 is significantly elevated in response to nutrient intake in absence of vagal signaling.

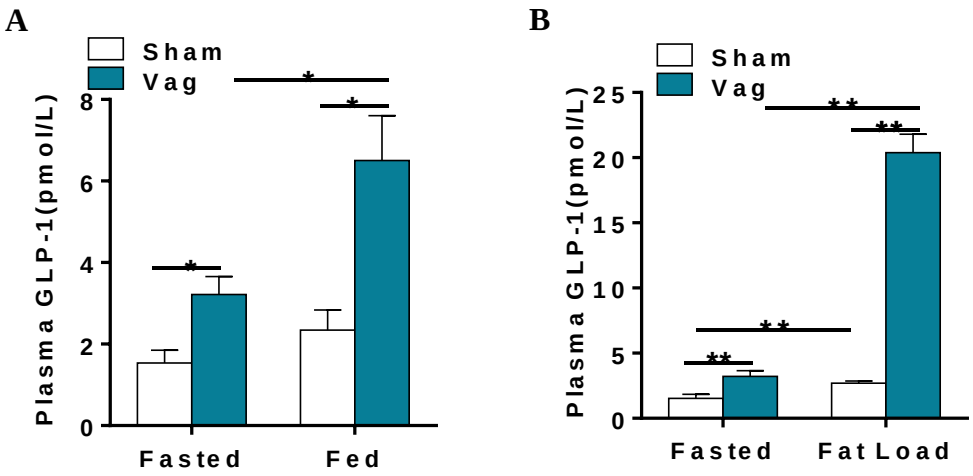



**Fig. 5:** Vagotomy significantly elevates GLP-1 in circulation. (A) Plasma GLP-1 levels in sham and vagotomized mice. (B) Plasma GLP-1 in sham and vagotomized mice at the fasted state or 1 h after administering 200 µl olive oil. Results are shown as mean± SEM, n=4-7/group. *P<0.05 and **P<0.01 versus control.

We then performed in vitro studies using rat hepatocytes to determine whether GLP-1 signaling alone is sufficient to inhibit hepatic lipid synthesis. We treated McA cells, a rat hepatoma cell line, with PA (50µM) in the presence or absence of exendin-4 (100 nM), a GLP-1 agonist, for 48 h. PA treatment significantly enhanced mRNA expression of SREBP-1c, a major transcription factor in hepatic lipid synthesis. Moreover, the expression of SCD-1, ACC and FASN, the downstream target genes of SREBP-1c, was significantly increased. Exendin-4, on the other hand, inhibited PA-induced expression of these lipogenic genes (Fig. 6). These findings were further corroborated by a significant reduction of secreted TG from palmitic acid (50 µM)-primary mouse cells when

incubated with exendin-4. Coincubation with exendin-4 lowered the secreted triglycerides almost to the level of the control (Fig. 7). No significant changes in secreted cholesterol was observed. Therefore, elevated GLP-1 in vagotomized mice may, at least partially, be responsible for reduction in TG.

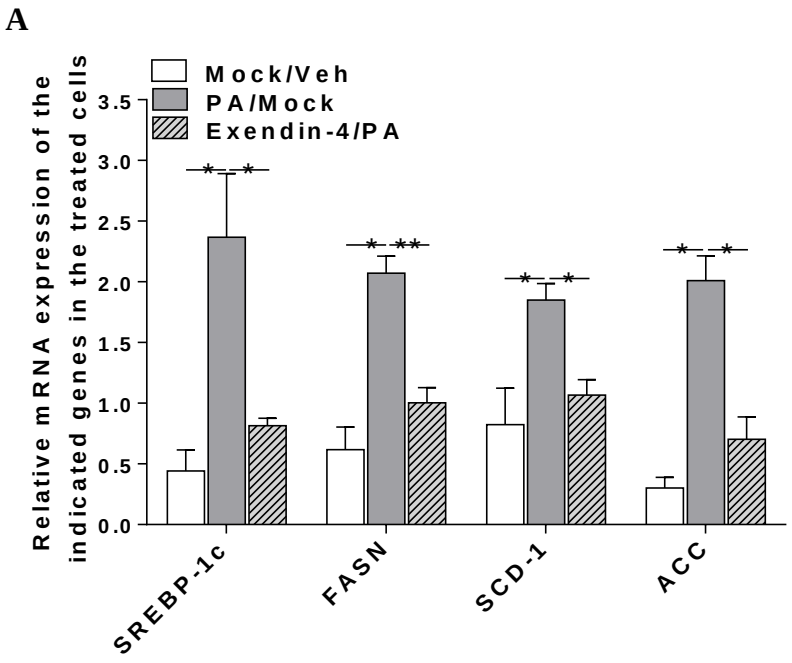



**Fig. 6:** Exendin-4, a GLP-1 agonist, inhibits lipid biosynthesis in hepatocytes in vitro. McA cells were treated with palmitic acid (PA) (50 µM) in the presence or absence of exendin-4 (100 nM) for 48 h. Relative mRNA expression of SREBP-c, FASN, SCD-1 and ACC was quantified in the treated cells by qRT-PCR. Results are shown as mean± SEM, n=4-7/group. *P<0.05 and **P<0.01 versus control.

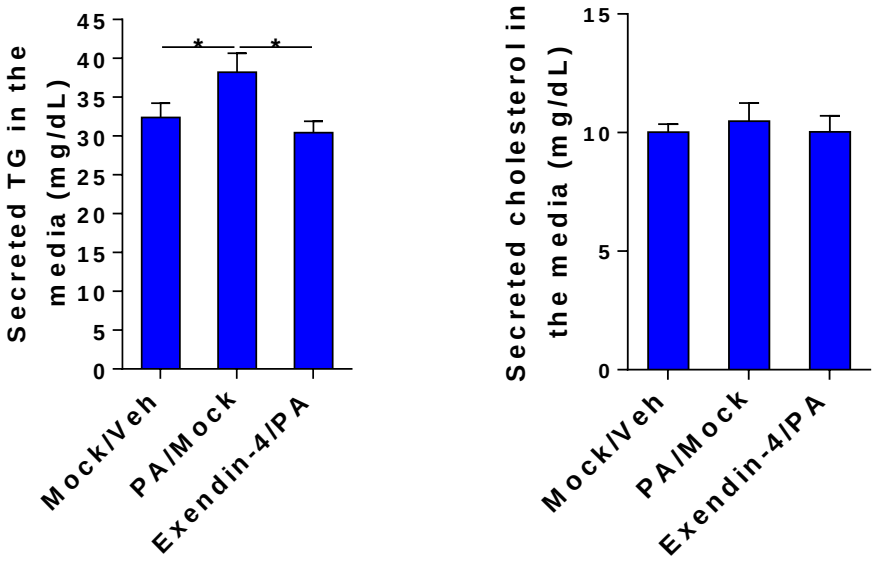
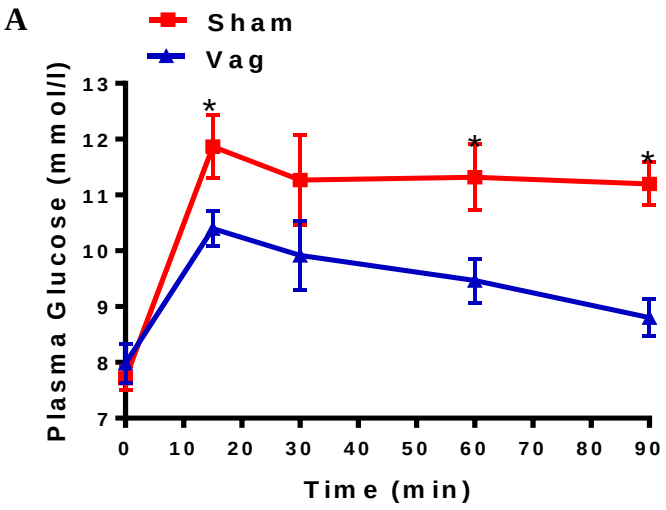



**Fig 7:** Exendin-4, a GLP-1 agonist, inhibits lipid biosynthesis in hepatocytes in vitro. Primary hepatocytes were treated with vehicle (0.5% BSA) or palmitic acid (PA) (50 µM) with or without exendin-4 (100 nM) for 48 h. TG and CHOL levels in the culture media was measured. Results are shown as mean± SEM, n=4-7/group. *P<0.05 and **P<0.01 versus control.

### 3.1.5: Vagotomy Improves Systemic Glucose Tolerance and Enhances Insulin Sensitivity.

We also investigated if the elevated circulatory GLP-1 in vagotomized mice any effect on insulin sensitivity and glucose tolerance as we observed improvement in lipid profiles. Oral glucose tolerance test (OGTT) in chow-fed mice revealed that glucose tolerance was significantly improved in vagotomized mice as indicated by lower blood glucose levels at 15, 60, and 90 min post glucose administration (Fig. 8A). Similarly, vagotomy led to

improved glucose tolerance in HFD-fed mice indicated by reduced glucose levels at 30, 60, and 90 minutes after glucose administration. On the contrary, HFD diet led to insulin resistance in sham mice exemplified by elevated blood glucose levels within 90 min post glucose administration (Fig. 7B). Insulin sensitivity was evidently improved in HFD-fed vagotomized mice.



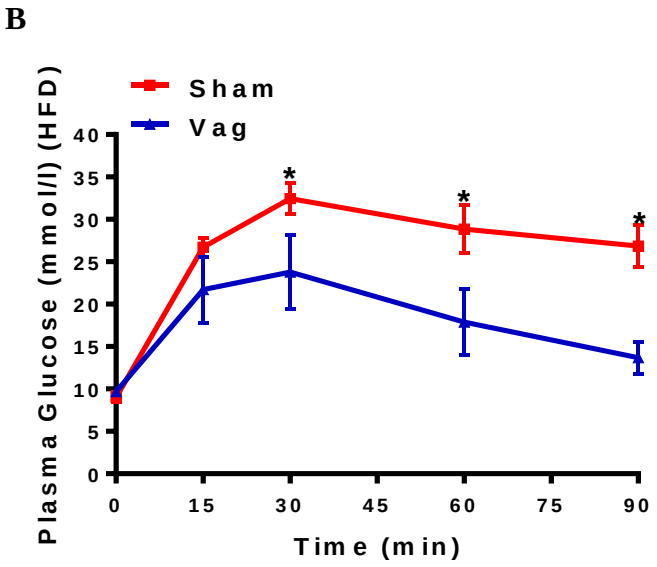
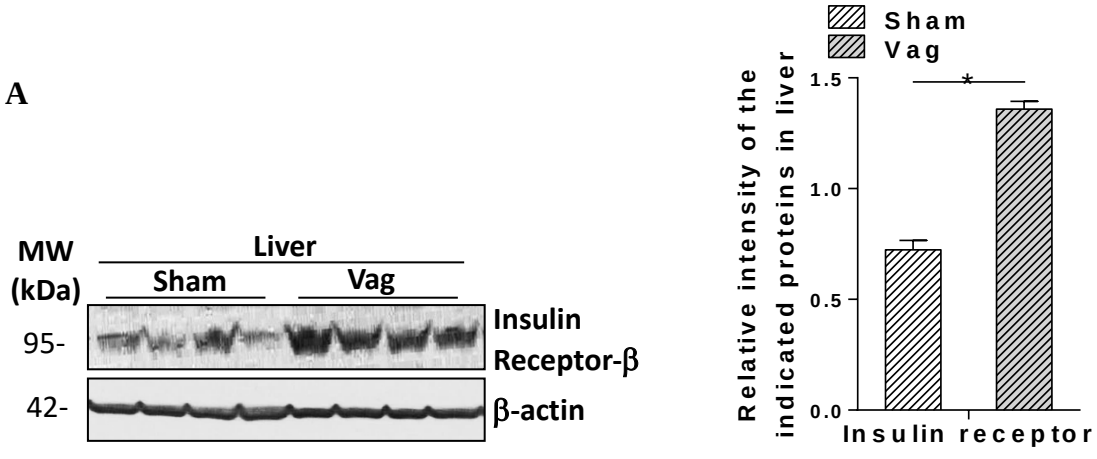



**Fig. 8:** Vagotomy improves systemic glucose tolerance and enhances hepatic insulin sensitivity. (A) Plasma glucose levels in sham and vagotomized mice at 0, 15, 30, 60 and 90 min after 12 hours of fasting and subsequent oral administration of glucose (2g/kg). (B) Plasma glucose levels in sham and vagotomized mice at 0, 15, 30, 60 and 90 min after 15 weeks of HFD feeding, 12 hours of fasting and subsequent oral administration of glucose (2g/kg). Results are shown as mean± SEM, n=4-7/group. *P<0.05 and **P<0.01 versus control.

To investigate the effect of vagotomy on insulin sensitivity in molecular levels, we investigated insulin signaling molecules in the liver by immunoblotting. The protein expression of the hepatic insulin receptor was found to be significantly increased compared to sham control mice (Fig. 9A). Moreover, injection of insulin into hepatic portal vein induced more profound activation of AKT, a key component in insulin signaling pathway, in vagotomized mice indicating enhanced insulin sensitivity in the liver (Fig. 9B). Taken together, these findings suggest that vagotomy improves glucose tolerance and insulin sensitivity in HFD-fed mice.



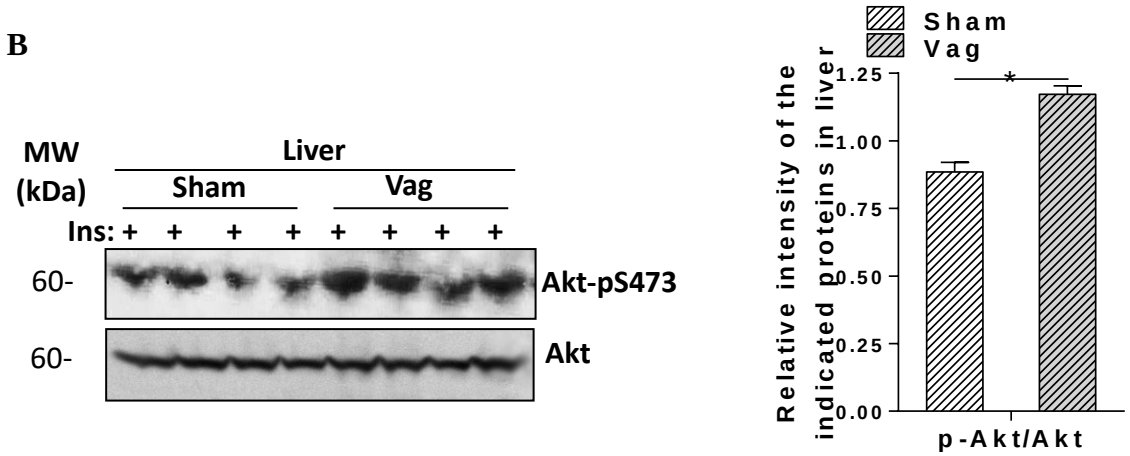



**Fig. 9:** Vagotomy improves hepatic insulin sensitivity by enhanced expression of insulin signaling molecules. (A and B) Immunoblotting analysis of protein levels insulin receptor and phosphor-Akt in the livers of sham and vagotomized mice. Results are shown as mean± SEM, n=4-7/group. *P<0.05 and **P<0.01 versus control.

## 3.2 Fenugreek

### 3.2.1 Fenugreek Improved Hyperlipidemia by Inhibiting VLDL Production and Secretion.

We first investigated the effect of fenugreek seeds on hepatic lipid and lipoprotein metabolism by quantifying the levels of mRNA expression of genes involved in fatty acid β-oxidation. We used CREBH-null mouse model to examine the effect of fenugreek on hyperlipidemia. Two groups of mice were fed either a control chow or chow containing

2% fenugreek seed meal for 7 weeks. The expression of PPARα, and its downstream target genes, CPT-1α, Acox-1 and MCAD in the liver was measured by q-RT-PCR. We observed significantly enhanced expression of the aforementioned genes in fenugreek-fed mice (Fig. 10). This indicates an improvement of fatty acid β-oxidation by fenugreek seeds. VLDL assembly and secretion are largely influenced by the availability of lipid substrates. Low availability of lipid substrates resulting from inhibition of lipid synthesis and augmented fatty acid β-oxidation may lead to lower VLDL synthesis.

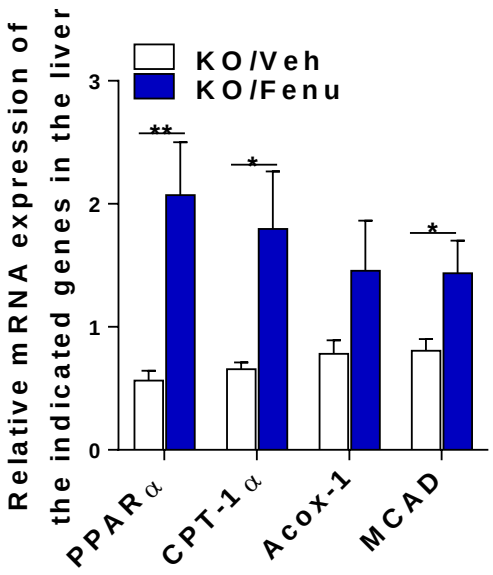


**Fig. 10:** Fenugreek seed improves hyperlipidemia by increased β-oxidation and VLDL synthesis. Two groups (n=5-6/group) of CREBH-null mice were subjected to chow diet with or without 2% fenugreek for 7 weeks. Plasma and livers were collected following 12 hours of fasting for the following analysis. Relative mRNA expression of PPARα, CPT-1α, Acox-1 and MCAD in the mouse liver was measured by q-RT-PCR. Results are shown as mean± SEM, n=5-6/group. *P<0.05 and **P<0.01 versus controls.

We then investigated if there were any changes in the plasma VLDL levels as they are involved in the transport of lipids from the liver to circulation for distribution to peripheral tissues and organs. Fenugreek feeding was also found to significantly reduce plasma VLDL levels which was exemplified by the reduced levels of apoB-100 protein in the plasma of the fenugreek-fed mice (Fig. 11). There was no change in the levels of apoE, another apolipoprotein, suggesting that fenugreek's inhibitory effect was primarily executed on VLDL-TG and VLDL-apoB metabolism (Fig. 11). The overall findings of this investigation suggest that fenugreek seed inhibited hepatic VLDL synthesis and secretion leading to ameliorated hyperlipidemia in the CREBH-null mice.

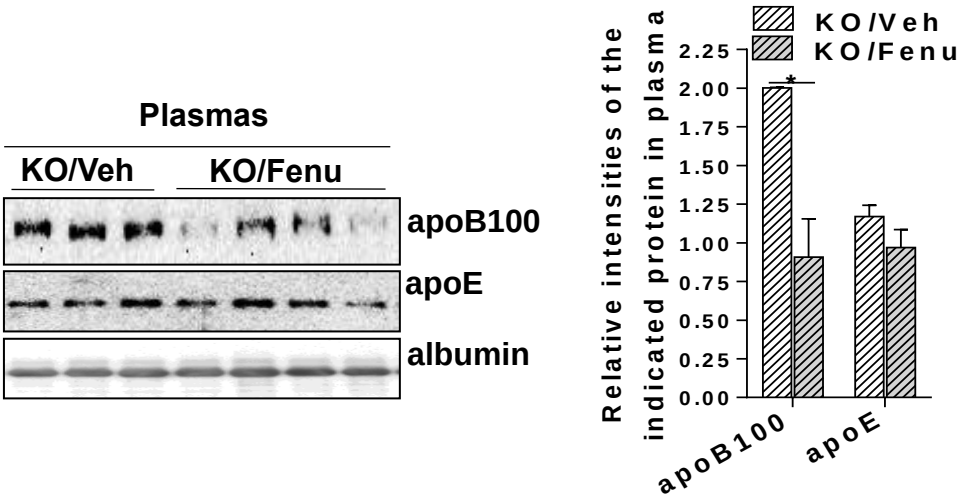



**Fig. 11:** Fenugreek seed improves hyperlipidemia by increased β-oxidation and VLDL synthesis. Two groups (n=5-6/group) of CREBH-null mice were subjected to chow diet with or without 2% fenugreek for 7 weeks. Plasmas were collected following 12 hours of fasting and used for immunoblotting. Immunoblot developed for apoB100 and apoE and immunoblots were quantified. Results are shown as mean± SEM, n=5-6/group. *P<0.05 and **P<0.01 versus controls.

### 3.2.2 Fenugreek inhibits hepatic SREBP-1c activity via enhanced Insig-1 and Insig-2 expression.

Depletion of CREBH in CREBH-null mice has been found to induce hepatic steatosis and hyperlipidemia via hyperactivation of SREBP-1c and hepatic de novo lipogenesis resulting from reduced Insig-2a expression [73]. The CREBH-null mouse model was, therefore, used in the present study to investigate the anti-hyperlipidemic effect of

fenugreek. The mRNA expression studies via RT-qPCR showed that fenugreek significantly induced the expression of Insig-1 and Insig -2b in the mouse liver compared to control mice (Fig. 12) This indicates as possibility of reduction in levels active of SREBP-1 as its activation is hindered by elevated Insig proteins.

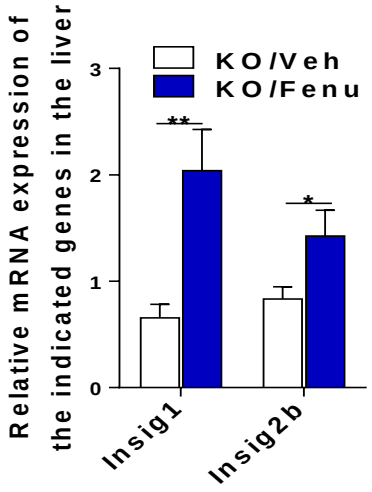
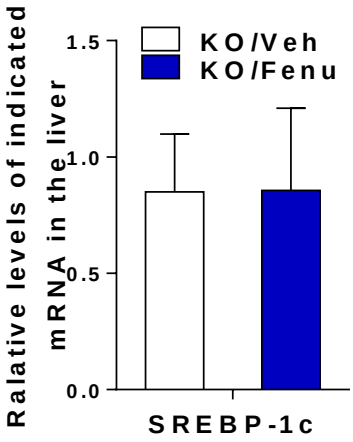



**Fig 12:** Fenugreek seed consumption enhanced Insig-1 and Insig-2 expression. Two groups (n=5-6/group) of CREBH-null mice were subjected to chow diet with or without 2% fenugreek for 7 weeks. Livers were collected following 12 hours of fasting and used for the analysis of relative mRNA expression of Insig-1 and Insg-2 by q-RT-PCR. Results are shown as mean± SEM, n=5-6/group. *P<0.05 and **P<0.01 versus controls.

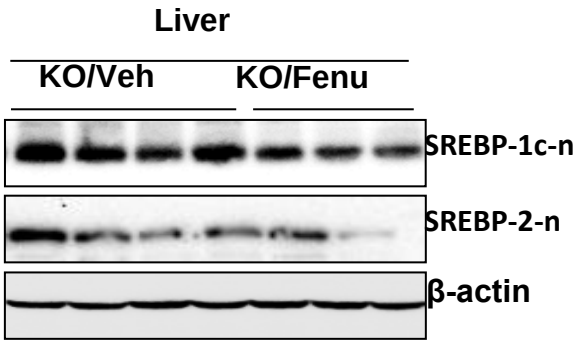
We followed up the investigation on transcription of Insig proteins with examining the impact of fenugreek seeds on SREBP signaling pathway. SREBP-1c mRNA expression studies showed no significant difference between the fenugreek-fed and the control mice (Fig. 13A). This suggests that fenugreek feeding has no influence on SREBP signaling

pathway, at least in the transcriptional level. Immunoblotting studies, however, revealed that fenugreek seed consumption reduced activation of SREBP-1c by proteolytic cleavage, indicated by reduced active form of SREBP-1c (N-terminal of SREBP-1) in the fenugreek-fed mice. SREBP-2 activation also showed a similar trend of reduction, but the change was not statistically significant (Fig. 13B). These observations were consistent with increased transcription of Insig-1 and -2., which may retain the SREBP-1c on the ER and prevent its activation by hindering translocation to Golgi apparatus

**A**




**B**



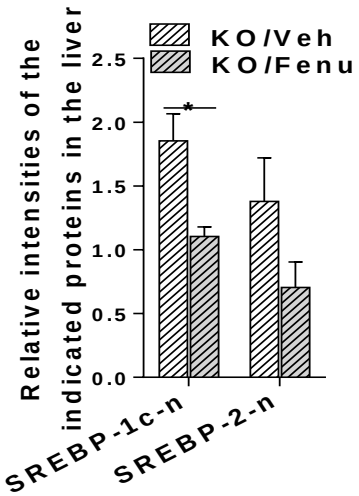



**Fig. 13:** Fenugreek seed inhibits activation of SREBP-1c. Two groups (n=5-6/group) of CREBH-null mice were subjected to chow diet with or without 2% fenugreek for 7 weeks. Livers were collected following 12 hours of fasting and used for the following analyses. (A) mRNA expression of SREBP-1c in the liver. (B) Immunoblotting analysis of n-terminal SREBP-1c and SREBP-2. Results are shown as mean± SEM, n=5-6/group. *P<0.05 and **P<0.01 versus controls.

We subsequently investigated the effect of inhibition of SREBP-1c activation on the mRNA expression of its target genes associated with hepatic lipid synthesis. The mRNA expression of the enzymes FASN, and SCD-1 was significantly reduced in fenugreek-fed mice (Fig. 14). This is consistent with the inhibition of SREBP-1c activation by Inig-1 and -2 hyperactiavtion. These findings further substantiate the possible inhibitory effect of fenugreek on de novo lipid synthesis in the liver. All these findings suggest that consumption of 2% fenugreek prevents SREBP-1c inhibition and hepatic de novo lipid synthesis by upregulation of Insig-1 and Insig-2.

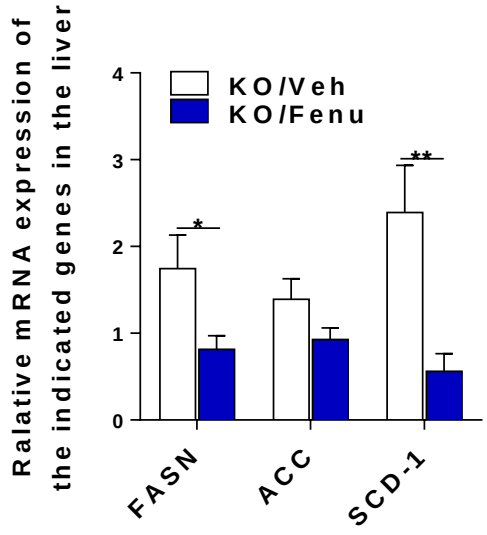


**Fig. 14:** Fenugreek seed hinders mRNA expression of enzymes involved in de novo lipid synthesis. Two groups (n=5-6/group) of CREBH-null mice were subjected to chow diet with or without 2% fenugreek for 7 weeks. Livers were collected following 12 hours of fasting and livers were collected. Liver samples were used for analysis of relative mRNA expression of FASN, ACC and SCD-1. Results are shown as mean± SEM, n=5-6/group. *P<0.05 and **P<0.01 versus controls.

### 3.2.3 Trigonelline Inhibits Hepatic Lipid Secretion.

Trigonelline is one of the major chemical components in fenugreek seeds. We employed cell-based culture systems to investigate if trigonelline is one of the bioactive compounds responsible for beneficial effect of fenugreek on lipid metabolism. To examine the effect of trigonelline, McA cells were treated with the saturated fatty acid, palmitic acid (PA) with or without coincubation with trigonelline. Before treating with PA, McA cells are pretreated with trigonelline (100µM) or mock ($H_2O$) for 24 h. The cells were subsequently incubated with PA (250µM) for an additional 6 h. Secretion of TG was significantly reduced in trigonelline treated cells (Fig. 15A). However, the CHOL levels were comparable between groups (Figure 15B). This finding suggests that trigonelline can effectively ameliorate lipotoxicity resulting from oxidative stress induced by saturated fatty acid. This also exhibits the active role of this bioactive compound in lipid metabolism regulation.

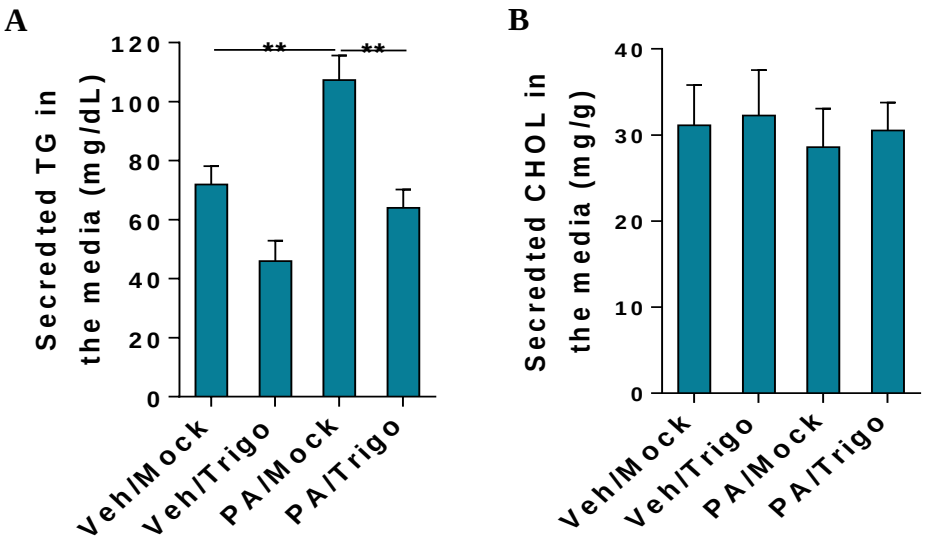



**Fig. 15:** Trigonelline protects from hyperlipidemia by reducing plasma TG levels. McA cells were treated with 100 μM of trigonelline for 24 h followed a 12 h fast. Cells were then treated with vehicle (BSA) or PA at the presence of mock ($H_2O$) or trigonelline (100μM) for additional 6 h. The media were collected for lipid extraction as descripted in Materials and Methods and used to measure (A) secreted TG, and (B) Secreted CHOL. For in vitro study, the data represent one out of three independent experiments (n=6/group). Results are shown as mean ± SEM. *P < 0.05 and **P < 0.01 versus controls.

## Chapter IV: Discussion

The goal of the vagotomy study was to delineate the underlying mechanism of sub-diaphragmatic vagotomy in obesity, hyperlipidemia and insulin resistance prevention. We demonstrated that complete disruption of the afferent and efferent fibers of the adnominal vagus nerve significantly decreases fasting and postprandial TG levels and VLDL biogenesis. Vagotomy also leads to elevated circulatory GLP-1 levels which may result in reduced hepatic fatty acid synthesis and VLDL production. HFD-induced hepatic steatosis and hyperlipidemia were also ameliorated after surgical removal of the vagus nerve. The vagotomized mice also exhibited improved hepatic insulin sensitivity and whole body glucose tolerance.

The autonomic nervous system has been implicated in the regulation of hepatic energy metabolism based on certain evidences. Increased vagal nerve activity by portal or jejunal infusion of lipids indicates possible role of vagus nerve in transmission of hepatic signals to the CNS [75]. Besides, infusion of long-chain fatty acids into hepatic portal vein increased hypertension [76, 77], and elevated circulatory epinephrine and norepinephrine [78], which suggests CNS activity in response to the infusion [79]. Vagus nerve has been reported to suppress systemic inflammation, modulate CNS responses to glucose and fatty acids, and regulate insulin sensitivity and hypertension [79-81]. The complete disruption of sub-diaphragmatic vagus nerve enabled us to study the local regulation of intrinsic gut-liver circuit in the absence of CNS regulation. We observed significant reduction in circulatory and hepatic TG levels in vagotomized mice. This reduction can be attributed to elevated GLP-1 levels since we demonstrated that GLP-1 can sufficiently downregulate genes involved in hepatic lipid synthesis, including SCD-1 and FASN, in

vitro. The in vivo reduction in SCD-1 and FASN can, therefore, be linked to increased GLP-1 levels in circulation. Reduced hepatic lipid synthesis in vagotomized mice led to less availability of lipid substrates for VLDL assembly, which essentially reduction VLDL secretion and prevented HFD-induced hyperlipidemia. Taher et al. reported significant reduction in diet-induced VLDL overproduction and dyslipidemia when exendin-4 was intraperitoneally injected into Syrian golden hamster [82]. We also observed significant improvements in insulin sensitivity and glucose tolerance in vagotomized mice.

Bariatric surgery is an effective clinical procedure to cut down body weight and treat obesity related metabolic diseases [83]. A significant loss in weight is observed after the surgery which is believed to be due to increased secretion of gut peptides, especially GLP-1 [84, 85]. It is hypothesized that direct delivery of ingested food to distal gut after the gastric banding procedure leads to increased GLP-1 secretion from the L-cells [86]. One explanation of the facilitation of weight loss by GLP-1 is due to its ability to enhance satiety and delay gastric emptying [87, 88]. Another explanation of the action of GLP-1 is its ability to enhance insulin secretion by the pancreas [88]. We observed a significant increase in the circulatory levels of GLP-1 in vagotomized mice, which possibly ameliorated hyperlipidemia evident from reduced plasma TG and VLDL secretion.

Fenugreek has been traditionally used in different parts of the world for treatments of various human disorders, including diabetes and hypertension, besides its use as a spice and condiment in a variety of food preparations. However, the exact molecular mechanism underlying its medicinal properties has not yet been completely delineated. The lack of scientific evidence has limited the use of this important dietary plant as a

reliable source of phytoceutical. In the current study, we demonstrated that the whole grain of fenugreek and its derived bioactive compound, trigonelline prevented hyperlipidemia upregulating expression of Insig proteins, inhibiting hepatic VLDL biogenesis, and enhancing fatty acid β-oxidation,

Hyperlipidemia is one major risk factor in the pathogenesis of cardiovascular disease and metabolic disorders [89]. Overproduction of VLDL particles is often recognized as contributor of hyperlipidic conditions [90]. Boban et al. observed a decrease in VLDL biosynthesis and secretion when mice were fed galactomannon extracted from fenugreek [91]. Similarly, Naidu et al. reported decrease in plasma triglycerides and restoration of VLDL-C to near normal levels by administering diosgenin extracted from fenugreek to HFD-streptozotocin (HFD/STZ)-induced diabetic rats [89]. Sharma et al. reported a significant decrease in VLDL-TG in type I diabetic patients when administered a diet containing fenugreek seed powder [92]. However, the underlying molecular mechanism of fenugreek action on VLDL metabolism remained ambiguous. A fiber cocktail containing fenugreek seed powder reportedly ameliorated hyperlipidemia in rats through enhanced clearance of VLDL particles from plasma by upregulation of apo B, E receptors in hepatocytes [93]. In our study, we observed a significant reduction in apoB100, the primary apolipoprotein in VLDL, in the plasma of fenugreek-fed mice while no considerable alteration in apoE levels was noticed. This observation suggests the beneficial effects of fenugreek through regulation of VLDL assembly and secretion. We also observed improvement in lipid metabolism demonstrated by reduced secretion of TG in trigonelline-treated rat hepatoma cells. Hamden et al. observed a significant decrease in serum TG when trigonelline was administered to diabetic rats [94]. Similarly, a

significant reduction in TG in diabetic rats was observed by Zhou et al. [95]. All these observations indicate beneficial effects of fenugreek seeds and trigonelline administration in controlling hyperlipidemia.

SREBPs are a family of transcription factors that control the expression of an array of enzymes for biosynthesis of cholesterol, fatty acids, and TG in hepatocytes [95-97]. SREBP activation is regulated by Insig (Insig1 and Insig2) and SCAP proteins within the ER membrane [98, 99]. The sterol-sensitive SCAP is associated with inactive SREBP on one side and with Insig on the other [100]. When cholesterol is abundant in ER membranes, SCAP undergoes a conformational changes enabling the binding between SCAP and Insig. This binding prevents the translocation of SCAP-SREBP complex to Golgi complex essential for SREBP activation. Low sterol levels lead to conformational changes in the SCAP, which leads to dissociation of SCAP-SREBP complex from Insig proteins [97, 101]. The SCAP-SREBP complex subsequently translocate to Golgi apparatus, where SREBP is cleaved by Site-1 protease (S1P) and Site-2 protease. The N-terminal domain generated by proteolysis leaves the membrane and moves to the nucleus, where it enhances transcription of genes required for de novo lipogenesis [101, 102]. Upregulation of SREBPs, especially SREBP-1c, has been recongnized to play a central role in the development and progression of metabolic syndrome. This warrants development of strategies to reduce the activity of SREBP-1c as a potential treatment [103]. Chemical inhibitors have been successfully used to hider SREBP activation. However, naturally occurring bioactive compounds which can interfere in SREBP activation is of great interest as chemical inhibitor may induce certain side effects. Our present study showed that administration of fenugreek seed inhibited activation of

SREBP-1c signaling by upregulating Insig1 and Insig2. This consequently reduced de novo lipogenesis and VLDL secretion from hepatocytes leading to improved hyperlipidemia in CREBH-null mice. It was previously reported that betulin, a triterpene isolated from birch bark, can inhibit the activation of SREBPs by inducing interaction between SCAP and Insig1 [104]. This inhibitory action of betulin resulted in decline in cholesterol and fatty acid biosynthesis thereby ameliorating HFD-induced obesity and improving insulin sensitivity. In another report, genistein, a phytochemical in soy, suppressed lipogenic gene expression in HepG2 cells via downregulation of S1P expression, which subsequently hindered proteolytic activation of SREBP-1 [105]. However, more studies are required to delineate the precise mechanism of fenugreek seeds on SREBP activation and fatty acid biosynthesis.

The novel findings from our study suggest that vagotomy can ameliorate HFD-induced hyperlipidemia, and improve insulin sensitivity. This study provides insights into developing vagotomy-based clinical approaches to treat metabolic disorders like diabetes and obesity. Our study also provided an insight into the mechanism of TG-lowering activity of fenugreek which alleviates hyperlipidemia. Observations from the fenugreek study would advance our understanding of the role of fenugreek in regulation of lipid metabolism and help conceive possible therapeutic implications. This study provides a rationale to develop of neutraceuticals from fenugreek seeds to treat metabolic disorders.

**Chapter Five: References**

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