STUDIES ON THE INFLUENCE OF SESAME OIL, GARLIC AND ONION ON CHOLESTEROL AND BILE METABOLISM

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In

BIOCHEMISTRY

by

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November-2007

DECLARATION

I hereby declare that the thesis entitled "**Studies on the influence of sesame oil, garlic and onion on cholesterol and bile metabolism**" submitted to the University of Mysore, for the degree of **Doctor of Philosophy** is the result of work carried out by me under the guidance of **Dr. K. Sambaiah**, Scientist - F, Department of Biochemistry and Nutrition, Central Food Technological Research Institute, Mysore – 570 020, during the period 2003 – 2007.

I further declare that the results have not been previously submitted for any other degree or fellowship.

Place: MYSORE

Date: November, 2007 **S. VIDYASHANKAR**

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November, 2007

CERTIFICATE

This is to certify that the thesis entitled "**Studies on the influence of sesame oil, garlic and onion on cholesterol and bile metabolism**" submitted by **Mr. S. Vidyashankar** to the University of Mysore, for the degree of **Doctor of Philosophy** is the result of work carried out by him under my guidance and supervision in the Department of Biochemistry and Nutrition, Central Food Technological Research Institute, Mysore - 570 020 during 2003 - 2007.

K. SAMBAIAH

..... to Minchu

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Abbreviations

°C	Degree Celsius
µg	Microgram (s)
µg/mL	Microgram/milliliter
µl	Microlitre (s)
µm	Micrometre
<	Less than
>	Greater than
ADP	Adenosine diphosphate
ALAT	Alanine amino transferase
Apo-AI	Apolipoprotein AI
Apo-AII	Apolipoprotein AII
ASAT	Aspartate amino transferase
b.w	Body weight
BA	Bile acids
BSA	Bovine serum albumin
C	Cholesterol
C/PL	Cholesterol:phospholipid ratio
CCK	Cholecystokinin
CE	Cholesterol esters
CE:FC	Cholesterol esters:free cholesterol ratio
CGS	Cholesterol gallstone
CHD	Coronary heart disease
cm	Centimeter
CoA	coenzyme-A
Con	Control
Con-A	Concanavalin-A
CSI	Cholesterol saturation index
CVD	Cardio vascular disease
dL	Decilitre (s)
DTNB	Dithio nitro benzoic acid
EDTA	Ethylene diamine tetra-acetic acid
FC	Free cholesterol
FER	Food efficiency ratio
Fig	Figure

g	Gram (s)
g/dL	Gram (s)/deciliter
g/Kg	Gram/kilogram
GNO	Groundnut oil
HCD	High cholesterol diet
HDL	High density lipoprotein
HI	Hydrophobicity index
HMG-CoA	3-hydroxy-methyl-glutaryl
HMW	High molecular weight
HPG	Heat processed garlic
HPLC	High Performance liquid chromatography
HPO	Heat processed onion
H	Hour (s)
I_c	Crystal index
Ig A	Immunoglobulin-A
Ig M	Immunoglobulin-M
I_g	Growth index
Ip	Intraperitoneal
I_t	Time index
IU	International unit
KD	Kilo daltons
Kg	Kilogram (s)
L	Litre (s)
LDH	Lactate dehydrogenase
LDL	Low density lipoprotein
LG	Lithogenic
LMW	Low molecular weight
M	Micelles
M	Molar
mA	Milli amperes
mg	Milligram (s)
mg/g	Milligram/gram
mg/mL	Milligram/milliliter
Min	Minute (s)
mL	Millilitre (s)
ml/h	Milliliter/hour
ml/min	Millilitre/minute

mM	Millimolar
MW	Molecular weight
N	Normal
NT	Nucleation time
NAD	Nicotinamide adenine dinucleotide
NaN_3	Sodium azide
ND	Not detected
nm	Nanometer
PAS	Periodic acid Schiff
PE-10	Polyethylene-10
PL	Phospholipids
RG	Raw garlic
RO	Raw onion
rpm	Revolutions per minute
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SFO	Sunflower oil
SO	Sesame oil
SOD	Superoxide dismutase
STDC	Sodium tauro deoxycholate
S-V	Small vesicles
TBS	Tris-buffer saline
TCDA	Taurochenodeoxy cholic acid
TD	Test diet
TG	Triglycerides
TL	Total lipids
UV	Ultraviolet
V/V	Volume/volume
W/V	Weight by volume
W/W	Weight by weight
Wt	Weight

Introduction

Dietary fat is an important source of metabolic energy. They are generally made up of different fatty acids, which are esterified with glycerol. The physical and nutritional properties of dietary lipids are largely influenced by the nature of their constituent fatty acids. In few vegetable oils, the non-saponifiable fraction of the oil also plays an important role in various physiological activities. The dietary lipids are digested in the presence of bile, which is secreted by the liver into the intestine. Bile emulsifies the fat and makes it available for action of lipases in the intestine. The resulting fatty acids and glycerol are absorbed from the intestine and transported to the liver and other tissues, where they are used up for different purposes.

The polyunsaturated fatty acid (PUFA) plays an important role in determining cholesterol level in serum. Sesame oil (SO) is traditionally accepted to be very stable against oxidative deterioration and in combating various degenerative diseases. This property of SO is attributed to the presence of characteristic lignans such as sesamin, episesamin, sesamol and sesamolin in the unsaponifiable fraction of sesame oil. These lignans have been shown to be hypocholesterolemic, hypolipidemic and antioxidant in nature. Much attention has not been paid to the beneficial effects of sesame oil on nutritional aspects.

Bile is an important means through which cholesterol is excreted. Saturation of cholesterol in the bile leads to the formation of cholesterol gallstones (CGS). Gallstone disease is a major hepatobiliary disorder, which is characterized by the formation of stones in the gallbladder. The blockage of bile duct and cystic duct by gallstones leads to several complications. Gallstone treatment is complicated because of difficulty in early diagnosis. Hence, dietary intervention to overcome the CGS has a wide scope in medicine. Spices are important food adjuncts being used since time immemorial and valued mostly for their organoleptic properties. Recently several beneficial physiological effects of spices have been documented. Some of these include: hypolipidemic influence, antidiabetic potency, antioxidant property, anti-inflammatory properties etc,.

Garlic and onion are widely consumed in the food preparations and have been proved to have wide physiological benefits to overcome many health disorders and they have been shown to possess hypolipidemic and hypocholesterolemic activities in both experimental animals and humans. During cooking foods are subjected to various

processing conditions like frying, boiling, heating etc., before it is consumed, which could change the physical and chemical composition of the ingredients.

The specific objectives of the current investigation are:

1. To study the effect of sesame oil on cholesterol metabolism in normal and hypercholesterolemic condition.

2. To study the antioxidant and antilithogenic potential of sesame oil.

3. To study the effect of raw and heat processed garlic, onion and sesame oil on cholesterol gallstone induction.

4. To study the effect of raw and heat processed garlic, onion and sesame oil on pre-established cholesterol gallstones.

5. To study the effect of bile from raw and heat processed garlic, onion and sesame oil fed animals on supersaturated model bile and cholesterol carriers in cholesterol gallstone formation.

The thesis is arranged in the sequence of 8 chapters. The **Chapter I** starts with the general introduction to diet and its constituents. A special emphasis is given to dietary lipids (fatty acids), their classification on the basis of their chain length, structure and nutritional and health benefits. The absorption, assimilation and transport of lipids in the system are discussed followed by nutritional importance. A brief concept of different diseases pertaining to lipid metabolism is reviewed under the sub-heads atherosclerosis, cardiovascular diseases and hepato-biliary diseases. The bile composition, formation, transport of biliary lipids in the bile and its functions has been given importance in the preliminary introduction to the bile. The types of gallstones in humans were discussed and the cholesterol gallstone disease is discussed elaborately. It included different stages involved during formation of cholesterol gallstones. The cholesterol gallstone markers such as cholesterol saturation index, hydrophobicity index of bile was discussed in deciding the lithogenicity of bile. The factors influencing the formation of gallstones were discussed to its relevance and importance. In this context both procrystallizing and anticrystallizing factors were discussed. The diagnosis of the CGS by various different techniques was high-lightened, followed by different treatments available in the medicine was taken into consideration. The dietary intervention may be one of the alternatives available for the

treatment of cholesterol gallstones since many dietary components like energy intake, carbohydrates and refined sugars, fatty acids, vegetable fibre have been shown to play a role. In this context, a brief introduction to spices and their importance and physiological effects were discussed. The chapter ends with a note on the hypocholesterolemic effect of garlic, onion and sesame oil and rationale for their selection to study their effects on cholesterol metabolism and gallstone formation and regression.

The **chapter II** gives the experimental design and protocols, of the various experiments, which were carried out in the subsequent chapters. In this chapter a brief protocol of all the procedures carried out throughout the investigation has been given with appropriate references.

The hypocholesterolemic and antioxidant effects of sesame oil is studied and presented in **chapter III.** This chapter begins with a brief introduction to the sesame seed and sesame oil and its health beneficial effects. The major unsaponifiable constituents such as: sesamol, sesamin, sesamolin etc., contents and their role in physiological conditions were briefed. The effect of sesame oil feeding at 10% on lipid metabolism was studied in male Wistar rats. The fatty acid composition of the sesame oil and its unsaponifiable constituents were analysed. The effect of sesame oil feeding on growth, serum and liver lipids was studied with respect to distribution of cholesterol between lipoproteins. The role of sesame oil on cholesterol absorption, excretion and denovo synthesis was studied. In this context, the activity of HMG-CoA reductase, the rate limiting enzyme in the cholesterol biosynthesis was measured. The absorption of cholesterol in high cholesterol diet fed group was studied by considering the amount of cholesterol intake and excreted in feces. The effect of sesame oil on bile secretion and composition was examined in rats by collecting bile after cannulating the common bililary-pancreatic duct. The antioxidant effect of SO on ferrous sulphate induced lipid peroxidation in rats was studied. The antioxidant enzymes such as: superoxide dismutase, catalase, glutathione peroxidase activities were measured. The vitamin E levels was also estimated. The activities of lactate dehydrogenase, alanine aminotransferase and aspartate aminotransferase were estimated in serum to analyze the extent of damage induced by peroxidation. The chapter was concluded by discussion of the results obtained.

The importance of bile and its constituents in the formation of cholesterol gallstones and effect of feeding spices (garlic and onion) and sesame oil was studied in **chapter IV.** This chapter starts with a brief introduction to the cholesterol gallstone pathogenesis and their causative factors. The pathologic conditions that generally precede the occurrence of CGS such as: lithogenic bile, gallbladder stasis and short nucleation time were discussed. The health beneficial effects of different spices with respect to lipid metabolism are given. In the results section, the induction of cholesterol gallstones by feeding lithogenic diet for different time interval was studied and the conditions for CGS induction was optimized. The effect of spices and SO was studied during the formation of CGS for 10 weeks. The different parameters like serum, liver and biliary lipids were analyzed. The cholesterol saturation index and hydrophobicity index of the bile was calculated. The hepatic lipid regulatory enzyme activities were studied. The chapter ends with the discussion.

The **chapter V** is focused on the effect of spices and sesame oil on the regression of pre-formed cholesterol gallstones in mice. This chapter begins with the introduction to the formation of cholesterol gallstones and the transport of lipid carriers in the bile. The existing treatment for cholesterol gallstones is discussed briefly followed by the dietary intervention and its effects in reducing the gallstone ailment. The experiments were designed at two intervals [short-term (5 weeks) and long-term (10 weeks)] of time to study the effect of spices and SO on existing cholesterol gallstones. The different parameters related to the CGS pathogenesis like CGS score, CSI and HI were determined at both the intervals. The serum and liver lipids were also analysed at both intervals. The hepatic lipid regulatory enzyme activities were also determined at both intervals. The gallbladder bile was subjected to gel permeation of chromatography and the resolved three fractions like micellar fraction, small vesicular and vesicular fractions were analysed for cholesterol, phospholipid and protein content and the amount of lipids carried by different fractions were also determined. The chapter was concluded with the elements of result-oriented discussion.

The formation of cholesterol gallstones in the gallbladder is controlled by various factors and designated as pro-crystallizing and anti-crystallizing factors present in the bile. These factors include mucus glycoprotein, calcium ions, bilirubin and low molecular weight proteins. The **chapter VI** is aimed at studying the role of proteins isolated from the hepatic bile of rats and their role in formation of cholesterol gallstones in model bile. This chapter begins with the note on different factors involved in the formation of cholesterol gallstones with more emphasis on the role of biliary proteins in

the formation of CGS. In the experimental part, the rats were fed with spices or SO for 8 weeks and the hepatic bile was collected by cannulation and the bile was studied for nucleation at different time intervals. The hepatic bile was concentrated and subjected to gel permeation chromatography for the separation of high and low molecular weight proteins. These HMW and LMW proteins were studied for their effect on cholesterol nucleation time in model bile. Further, the LMW protein was subjected to lectin affinity chromatography using Con-A and the bound and unbound fractions were studied for cholesterol nucleation time in model bile. The different crystal parameters like crystal growth index (I_g), crystal index (I_c) and time index (I_t) were calculated. The bound protein fraction was subjected to SDS PAGE and the molecular weight of the protein was determined. The chapter concludes with discussion.

The summary and conclusions of the present findings were given in the **chapter VII** and the thesis concludes with the bibliography **chapter (VIII)** arranged alphabetically in sequential order.

Signature of the candidate
(S. Vidyashankar)

Signature of the guide
(Dr. K. Sambaiah)

Chapter – I

Introduction

The essential components of a diet are: carbohydrate, protein, fat, vitamins, minerals and water. Carbohydrate, protein and fat yield energy, provide for the growth and maintainance of tissue subjected to wear and tear. Carbohydrate and protein yield 4 Cal/g energy compared to fat 9 Cal/g. Apart from this, these nutrients also support structural components of the body, and provide substances that regulate physiological processes. The fat and oil are synonymous where in, the former exists as solid and the later as liquid at room temperature. Dietary fat (vegetable or animal origin) consists of mainly triglycerides and other minor lipids like phospholipids, free fatty acids, glycoplipids, sulpholipids and phytosterols. Natural fats and oils vary in their nutritional and physical properties depending on the amount and type of fatty acids attached to the glycerol backbone. Fat also contributes to the palatability and flavor of food hence one can rejoice eating. Adipose tissue, which is the repository of most of our body fat, serves as an energy reservoir, as a heat conservator, and as a shock absorber. Fat is also the transport vehicle for vitamins A, D, E and K.

Apart from these, the animal fat in addition to the previously mentioned components also contains cholesterol, which has absorbed the brunt of the anti-fat attack, is a compound that is required for life. It is not essential in the sense of essential fatty acids, since the body can synthesize it, but is crucially important component of our biological economy. Cholesterol comprises about 0.2% of normal body weight. Most of it (about 33%) is in the brain and the nervous system, where its function has been probed beyond suggesting that its major activity is as an insulator. Almost another one-third of body's cholesterol is in muscle, where it is a structural component. Cholesterol is the parent substance for vitamin D_2, bile acids, adrenocorticoid hormones and sex hormones. Thus, it is one of the important biological substances. Similarly, the plant lipid also contains various minor components including phytosterol, which play a major role in the various physiological processes.

Triglycerides

Triglycerides are the major components of the lipids in edible fats and oils. They are the esters of fatty acids with glycerol.

Fatty acids and their types

Fatty acids are components of triglycerides, phospholipids, and cholesterol esters and also have structural, energetic and metabolic functions. Fatty acids are distinguished from each other on the basis of their chemical structure. All have a chain like structure with an acid or carboxyl group (OH-C=O) at one end and a methyl group (CH_3) at the other. The rest of the molecule consists of a hydrocarbon (CH_2) chain varying in length from 2 to 20 or more carbons. The most common fatty acids in foods have an even number of carbon atoms ranging from 12-22 carbons, though shorter, longer, and odd-numbered fatty acids also exist.

Fatty acids serve at least three vital functions: A few, such as linoleic acid (LA), α-linolenic acid (LNA) are essential nutrients; others, particularly the short-chain fatty acids, provide energy; long-chain fatty acids are structural components of all cell membranes. Fatty acids seldom exist by themselves but are chemically incorporated into larger molecules such as triglycerides and phospholipids.

The main distinguishing features of fatty acids are -

Chain length

Short-chain fatty acids have fewer than 8 carbon atoms; medium-chain fatty acids have 8-12 carbon atoms, whereas those with more than 12 carbons are considered long chain fatty acids. Short-chain fatty acids are water soluble and absorbed directly from the intestine into the blood stream. They are usually metabolized for immediate energy needs and are not abundant in most foods.

The long-chain fatty acids with 14 or more carbons have received considerable attention from nutrition and health point of view. These are stored in membranes as components of phospholipids and in adipose tissue as triglycerides. In addition to providing energy, long-chain polyunsaturated fatty acids are constituents of cell membranes where they are available as precursors for several metabolic pathways. They also have pronounced effect on protein function [Murphy, 1990].

Saturation

Besides chain length, the number of hydrogen atoms they carry distinguishes fatty acids. The saturated fatty acids contain the maximum number of hydrogen atoms they can hold. A fatty acid becomes unsaturated when a pair of hydrogen atoms is removed, thereby creating a double bond between the adjacent carbon atoms.

Specific enzymes carry out desaturation process but only at certain carbon sites. Thus the location of double bonds in a fatty acid is highly regulated.

Based on the unsaturation, fatty acids are classified into three categories: (i) saturated fatty acids, (ii) monounsaturated fatty acids and (iii) polyunsaturated fatty acids.

Saturated fatty acids (SFA)

Saturated fatty acids (SFA) chiefly comprise of butyric (C4:0), caproic (C6:0), caprylic (C8:0), capric (C10:0) which are termed as short chain fatty acids (SCFA), lauric (C12:0), myristic (C14:0) which are regarded as medium chain fatty acids (MCFA) and palmitic (C16:0) and stearic (18:0) acids are the major long chain fatty acids (LCFA). Other minor LCFA of dietary origin are arachidic acid (C20:0), behenic (C22:0), lignoceric (C24:0), cerotic (C26:0) and montanic acid (C28:0).

These fatty acids differ in chain length, absorption and metabolism. The fatty acids of C4:0 to C10:0 chain lengths are directly absorbed and transported via portal system to the liver and rapidly metabolized in that organ. MCFA and LCFA are absorbed as chylomicrons via lymphatic system and oxidized in mitochondria whereas fatty acids above C20:0 are first shortened in their chain length by peroxisomal β-oxidation, which differs from normal mitochondrial β-oxidation.

It has been shown that saturated fatty acids have hypercholesterolemic effect when compared to unsaturated fatty acids [Grundy and Denke, 1990]. Saturated fatty acids up to C10:0 chain length do not raise plasma cholesterol whereas lauric (C12:0), myristic (C14:0) and palmitic (C16:0) acids have generally been regarded as the choesterol enhancing fatty acids and the major plasma lipoprotein affected by these fatty acids is what is known as low density lipoprotein (LDL) [Ney, 1991]. Palmitic acid is quantitatively the most important since it is the principle saturated fatty acid present widely in animal and plant fats [Kinney, 1994]. Stearic acid is considered, as 'neutral fat' since it do not raise the plasma cholesterol level. Stearic acid is converted into oleic acid rapidly (Haumann, 1998; Bonanome and Grundy, 1988).

Monounsaturated fatty acids (MUFA)

The predominant MUFA are palmitoleic acid (C16:1, n-7), oleic acid (C18:1, n-9), vaccenic acid (C18:1, n-7), cetoleic acid (C20:1) and erucic acid (C22:1, n-9). Vegetable oils contain very little palmitoleic acid, except palmolein whereas olive and

rapseed oils are rich in oleic (C18:1) and erucic acids (C22:1) respectively [Basiron, 1996]. MUFA are considered as neutral with respect to their effects on serum cholesterol. Some studies showed that MUFA when substituted for SFA lowered plasma cholesterol levels as effectively as PUFA. The lowering of cholesterol was found to be almost entirely from that of LDL fraction. LDL enriched with oleic acid is resistant to oxidation [Mensink and Katan, 1989].

Polyunsaturated fatty acids (PUFA)

The unsaturated fatty acids with more than one double bond are generally classified as PUFA. The important dietary PUFA mostly belongs to two separate families, n-6 and n-3. Linoleic (n-6) and linolenic (n-3) are essential fatty acids and are not synthesized by mammals. Hence, they have to be supplied through the diet. These fatty acids differ in structure and metabolism and hence in their biochemical and physiological effects.

A number of studies have showed that when PUFA are substituted for saturated fatty acids in the diet, these lowered the plasma cholesterol, principally from the LDL fraction [Grundy and Denke, 1990]. HDL-cholesterol in not altered when the contribution of linoleic acid in the diet is not more than 12% of dietary energy or the ratio of PUFA/SFA does not exceed 1.0. Generally, dietary PUFA decreases plasma cholesterol by enhancing receptor mediated LDL uptake and by enhancing cholesterol excretion via bile acids [Spady and Dietschy, 1985]. In contrast, the dietary n-3 fatty acids are found to reduce the concentrtion of VLDL by lowering triacylglycerols. Very high intakes of fish oil rich in n-3 PUFA have been found to lower total and LDL-cholesterol [Kinsella, 1988].

Linoleic acid (n-6 family) is a precursor of arachidonic acid (20:4, n-6), component of skin acylglucoceramides, hypolipidemic when compared to saturated fatty acids and has essential fatty acid activity. The various functions attributed to n-3 family are hypolipidemic, essential for vision and neural membrane formation and reduce arachidonic acid synthesis [Kinsella, 1988]. Though PUFA beneficially influence blood lipid profiles, they induce oxidative stress and demand increased supply of antioxidants in the diet [Gurr, 1990].

Minor constituents in oil and fat

Apart from triglycerides and other major constituents, the minor constituents present in the unsaponifiable fraction of fat also contribute to the nutritional and health

effects. Butter and ghee contains vitamins A and D. All the vegetable oils contain various tocols (tocopherols and tocotrienols). Tocols are antioxidants and they protect the cell from free radical damage. The total tocol content of palm oil is 1g/100g, of which tocopherols and tocotrienols constitute 30 and 70% respectively. Rice bran oil has 500 mg/100 g of total tocols of which 1/3 are tocopherols and 2/3 tocotrienols. The tocotrienols are hypocholesterolemic due to their inhibitory effect on endogenous cholesterol synthesis and also have antioxidant, antithrombotic and anticarcinogenic properties [Rong et al, 1997]. The minor constituents present in sesame oil (sesamin, sesamol, episesamin and sesamolin etc.) also have antioxidant and hypocholesterolemic properties [Hirose et al, 1991]. Rice bran oil has a hypocholesterolemic effect due to the presence of tocotrienols and oryzanol content [Seetharamaiah and Chandrasekhara, 1989; Sugano and Tsuji, 1997]. Oils also contain plant sterols, which may be of nutritional significance.

Fat digestion and assimilation

Fat digestion begins in the mouth where a lingual lipase is secreted from the glands near the tongue. The hydrolysis of lipids takes place in the stomach at a pH of about 4.5 - 5.5. The main products of gastric digestion are SCFA and MCFA. The major site of lipid digestion is the upper part of the small intestine. Churning action of stomach creates coarse oil in water emulsion, which on entering the duodenum, mixes with bile, which supplies bile salts and phospholipids and pancreatic juice which in turn supplies lipase and other digestive enzymes.

Bile, which is synthesized and secreted by the liver, contains large quantities of bile salts mainly in the form of sodium salts, which are extremely important for the digestion of fat. Bile salts reduce the surface tension of the fat globules in the intestine. This property of the bile salt is helpful in breaking down the insoluble fat globules by agitation in the small bowel. The lipases (pancreatic and intestinal) of the intestine hydrolyse triglycerides into fatty acids, monoglycerides and glycerol.

Pancreatic lipase catalyzes the hydrolysis of fatty acids from position 1 and 3 of a triglyceride. Very little hydrolysis occurs at Sn-2 position. The enzyme attacks triglyceride at oil-water interface of large emulsions. Bile salt molecules accumulate on the surface of the lipid particles displacing other surface-active constituents and confer a negative charge on the oil droplets, attracting a small protein colipase to the surface. Thus, bile salts, colipase and pancreatic lipase interact on the surface to

Table 1.1: Fatty acid composition of edible fats and oils (%) from different sources

	SFA			MUFA	PUFA	Minor constituents
	SCFA	MCFA	LCFA			
Coconut	14	63	12	7	2	-
Palm kernel	7	65	10	15	2	-
Ghee	10	15	40	32	2	Vitamin A&D
Vanaspathi	nd	1	73	19	3	-
Red palm (raw)	nd	1	49	40	9	-
Palm	nd	1	44	44	10	Tocotrienols
Olive	nd	nd	13	76	10	-
Groundnut	nd	1	23	50	25	-
Sesame	nd	nd	15	42	42	Sesamol, sesamin
Rice bran	nd	nd	22	41	35	Tocotrienols, oryzanol
Cotton seed	nd	nd	21	25	52	-
Corn	nd	nd	12	32	55	-
Sunflower	nd	nd	13	27	60	-
Safflower	nd	nd	13	17	70	-
Soyabean	nd	nd	15	27	53	-

nd-Not detected,

SFA-Saturated fatty acid, SCFA - Short chain fatty acid,

MCFA-Medium chain fatty acid, LCFA-Long chain fatty acid,

MUFA -Monounsaturated fatty acid, PUFA - Polyunsaturated fatty acid.

Source: Ghafoorunissa [1994] The National Medical Journal of India, **7,** 270.

form a ternary complex, which also incorporates the calcium ions that are necessary for the enzyme activity [Gurr, 1997]. Hydrolysis of fatty acid at Sn-2 position of phospholipids is catalyzed by phospholipase A_2, present in pancreatic juice as an inactive proenzyme, which is converted to active form by release of a terminal peptide by trypsin. Pancreatic cholesterol ester hydrolase cleaves fatty acids from ingested cholesterol esters and retinyl esters. The rate at which pancreatic lipase acts depends on the physical and chemical characteristics of fatty acid. Longer the chain length, slower is the release of fatty acids from triglycrides. Hence, SCFA and MCFA are rapidly released [Gurr, 1997].

SCFA are absorbed across the gut wall as individual fatty acids. They enter the blood stream and rapidly utilized for energy production. Other fat digestion products are mixed with bile acids to form mixed micelles with a core of non-polar components and an outer shell of amphiphillic constituents. Bile acids are reabsorbed by enterohepatic circulation [Gurr, 1997].

Malabsorption can occur even when digestion is functioning normally due to defects in small intestine affecting absorption surfaces. This may occur during severe bacterial infections of gut, sensitization of the gut to dietary components such as gluten in celiac disease or allergens. The excretion of fat in faeces is then massively increased, resulting in the increased excretion of 10-hydroxy stearic acid formed by bacteria which is present in high concentration in stools. This condition is called steatorrhoea. The severe fat malabsorption causes essential fatty acid and fat-soluble vitamin deficiency [Gurr, 1997].

Transport of lipids

After absorption, lipids are transported in blood in combination with proteins (apoproteins) and these are commonly called lipoproteins. There are 4 major types of lipoproteins namely: chylomicrons, VLDL, LDL and HDL.

Chylomicrons are assembled in the intestinal mucosa, keep exogenous triacylglycerols and cholesterol suspended in aqueous solution. These lipoproteins are released into intestinal lymph and transported through lymphatic vessels. In the blood vessels, chylomicrons are converted to chylomicron remnants by the action of lipoprotein lipase of muscle and adipose tissue. Chylomicrons, therefore, function to deliver dietary triacylglycerols to muscle and adipose tissue and dietary cholesterol to liver [Smith et al, 1978].

Very low density lipoproteins (VLDL), which are synthesized and secreted by liver as lipid transport vehicle are also degraded by lipoprotein lipase. The VLDL remnants appear in circulation first as intermediate density lipoproteins (IDL) and then as low-density lipoproteins (LDL). In the transformation of VLDL to LDL, all its apoproteins except apo B-100 are removed and much of their cholesterol is esterified by HDL-associated enzyme lecithin: cholesterol acyltransferase (LCAT). The enzyme transfers a fatty acid molecule from Sn-2 position of lecithin to cholesterol with concomitant formation of lysolecithin [Smith et al, 1978].

Low-density lipoprotein (LDL) formed from VLDL is then taken up by liver via LDL receptor, a cell surface transmembrane glycoprotein, which specifically binds both apo B-100 and apo E. Thus LDL uptake is receptor-mediated endocytosis. In the liver cells, the LDL's apo B-100 is rapidly degraded to its component amino acids. The cholesterol esters are hydrolyzed to yield cholesterol, which is subsequently incorporated into the cell membrane. Any excess intracellular cholesterol is re-esterified for storage within the cell through the action of acyl CoA: cholesterol acyltransferase (ACAT), mainly with oleic acid. Since, LDL is involved in distributing cholesterol to various tissues, it is some times called 'Bad cholesterol'.

High-density lipoprotein (HDL) has essentially the opposite function of LDL. It removes cholesterol from various tissues and transports it to liver. The nascent HDL is synthesized and sectreted by the liver into blood where it is converted into mature HDL from the components largely obtained through the degradation of other lipoproteins. HDL removes cholesterol from cell surface membranes and converts it into cholesterol esters through the action of LCAT. HDL is thus functioning as a cholesterol scavenger and getting involved in reverse cholesterol transport. HDL brings cholesterol from various tissues to liver, which is the only organ capable of disposing significant quantities of cholesterol as bile acids. HDL is therefore, called 'Good cholesterol' [Barter and Rye, 1996; Fielding and Fielding, 1995].

Cholesterol

Cholesterol is derived from two sources (diet and endogenous synthesis) and is secreted into the bile and eliminated through feces. Dietary cholesterol was earlier reported to have no effect on serum cholesterol but now it is accepted that every 100 mg intake of dietary cholesterol increases 4-5 mg% of plasma cholesterol levels. Humans absorb 10-14%, while rats 50-80%, monkeys 40%, dogs 40-75% and rabbit's upto 90% of dietary cholesterol. With reference to the body weight, the

cholesterol absorption capacity of humans is only 1% of that of the animals mentioned above [Kansal, 1995]. Certain individuals respond strongly and others weakly to dietary cholesterol (hyper and hyporesponders). This phenomenon is seen in a variety of animals including man [Gurr, 1992]. Some epidemiological observations also suggest that there is a linear association between dietary and plasma cholesterol [Gurr, 1992]. According to WHO recommendations the maximum cholesterol intake is 300 mg/day. Hence it is advised to keep the intake as low as possible especially in people with risk factors to CVD [Ghafoorunissa, 1994].

Excretion of cholesterol

Majority of the cholesterol is eliminated from the body in the feces after conversion to bile salts. The remainder is excreted as neutral steroids. Much of the cholesterol secreted in the bile is reabsorbed, and is believed that the cholesterol, which serves as precursor for the fecal sterols, is derived from the intestinal mucosa. Coprostanol is the principal sterol in the feces and it is formed by intestinal bacteria. A large proportion of the biliary excretion of bile salts is reabsorbed into the portal circulation, taken up by the liver and resecreted in the bile. This is known as the enterohepatic circulation. The bile salts not reabsorbed or their derivatives are excreted in the feces.

Atherosclerosis

Atherosclerosis is a disease of the intima of arteries involving focal accumulation of lipids, complex carbohydrates, blood and blood products, fibrous tissue and calcium deposits and associated medial changes. It is a major cause for death from coronary heart disease. The injury to endothelium of arteries produced by chemical or mechanical action is a key element in the initiation of plaque formation [Gurr, 1992]. Hyperlipidemia is a major risk factor for atherosclerosis. Elevated LDL cholesterol plays an important role in the plaque formation.

Coronary heart disease (CHD)

Coronary heart disease is a major cause of death and ill health in developed countries. Hopkins and Williams [1981] identified 246 risk factors for CHD. The various risk factors for CHD included: elevated total blood cholesterol, LDL-cholesterol, low HDL-cholesterol, gender, advancing age, genetic predisposition, smoking, high blood pressure, obesity, physical inactivity, diabetes mellitus and elevated lipoprotein (a) [Lp (a)] levels.

Hepato biliary disorders

Bile plays an important role in the digestion and absorption of lipids and it is synthesized and secreted by the liver [Coleman, 1987; Gurantz and Hofmann, 1984]. About 500 mL of bile is secreted daily and this passes along the canalicular ducts, hepatic duct, cystic duct and finally to the gallbladder and intestine. In the gallbladder, the bile is concentrated and stored and is secreted into the duodenum in response to meal. The hepatic and gallbladder bile composition is given in Table –1.2.

Table 1.2: The composition of Hepatic and Gallbladder bile

Parameters	Hepatic bile	Gallbladder bile
Color	Goldern yellow orange	Dark brown to greenish brown
Water	95-97%	85-90%
Bile salts	15-50mM	150-400mM
Phospholipid	5-16mM	40-130mM
Cholesterol	2-5mM	10-30mM
Bilirubin	0.3-0.8mM	1.5-7mM
Proteins	250mg/dL	700mg/dL
Fatty acids	250mg/dL	350mg/dL
pH	7.0-7.8	6.9-7.0
HCO_3^-	20-30mM	<1M
C^{++}/Ca total	0.8-1.2mM/2-3mM	2-3mM/10-18mM
Osmolity	Similar to plasma	Similar to plasma

Source: www.uwgi.org/gut/liver

Formation of bile

Bile formation results from the active secretion of osmotically active compounds by hepatocytes into the canalicular space, followed by the passive movement of water through tight junctions. Bile salts are the main solutes in bile and are considered to be the major osmotic driving force in the generation of bile flow, although bile salt independent processes also contibute to bile production. Transport of biliary constituents from blood to bile is a vectorial process that include uptake from sinusoidal blood, intracellular transport through the hepatocyte (with or without metabolic modifications) and canalicular secretion against steep concentration gradients that require energy dependent active transport. Although the main determinants of overall bile flow is the volume of water generated at the canalicular level. Further modifications of the bile composition occur along the biliary tree. In addition, bile salts - the major biliary solute undergo enterohepatic cycle as a result of the presence of an active transport mechanism located in the apical pole of enterocytes at terminal ileum. This enables retrival of bile salts from the intestinal lumen and transport to the portal circulation and ultimately to the liver for uptake and re-secretion [Arrese and Trauner, 2003].

Bile is composed mainly of water (>90%) and is the primary excretory route for organic compounds such as cholesterol, lipid hormones, and drugs with low water solubility. The hepatocyte is the major site for cholesterol synthesis and peripheral uptake, and excess cholesterol is directly secreted into bile or converted into bile salts. Cholesterol and phosphatidylcholine are mainly secreted into bile as small unilamellar vesicles (40-200 nm in dameter) that form on the external hemi-leaflet of the canalicular membrane. Because of their detergent properties, bile salts secreted from hepatocytes within the canalicular lumen directly convert vesicles into smaller (40-100A° in diameter) structures called micelles. Thus mixed micelles containing bile salts and phospholipids and unilamellar vesicles are the physiological carriers of cholesterol in bile [Portincasa et al, 2006].

Bile secretion

There are two types of bile secretion by liver –

a. Bile acid – dependent bile flow which is driven by osmotic effects of bile acids
b. Bile acid - independent bile flow of which mechanism is not known.

Functions of bile

Bile plays an important role in many physiological processes like -

Emulsification

Bile salts are strong detergents which emulsify the fat in the intestine and aides in the absorption of fat digestion products and fat-soluble vitamins. Inadequacy of bile salts leads to indigestion and results in severe steatorrhea, a condition in which more than 50% of dietary fat appears in the feces.

Neutralization of acid

Bile helps to neutralize the highly acidic chyme, which decends from stomach and adjusts appropriately the pH for other intestinal enzymes to bring about digestion of food.

Excretion

Bile is an important vehicle for the excretion of cholesterol in the form of bile acids, bile pigments, several drugs, toxins, killed bacteria, inorganic ions and several other harmful substances which are not excreted by kidney, lungs or skin.

Any alteration in the bile composition in turn and its metabolism leads to the complicated gastroenterological problems such as jaundice, liver or biliary cirrhosis, pancreatitis, carcinoma of gallbladder, and gallstone pathogenesis.

Gallstone pathogenesis

Gallstone pathogenesis is a major hepatobiliary disorder characterized by the formation of stones in the gallbladder. It is an extremely common problem. There are approximately 500,000 new cases of symptomatic cholesterol gallstones in the US annually. The blockage of cystic duct or bile duct leads to several complications (Fig 1.1). Gallstones may be stuck at the junction where duodenal opening opens to small intestine (Fig 1.2). Presence of gallstones in the gallbladder may cause perforation in the gallbladder, which in turn lead to the leakage of gallbladder contents into the peritoneal cavity leading to several complications.

Three distinct types of gallstones can be distinguished according to their composition viz: (1) pigment gallstone or Ca^{2+} stones, (2) mixed or black stones which contain Ca^{2+} and polymerized bilirubin and related pigments throughout and (3) cholesterol gallsontes (CGS)

Cholesterol gallsontes

Cholesterol gallstones (CGS) are generally composed of 90-96% cholesterol. Prevalence of CGS is > 90% of different types of gallstones when considered together. It is a result of complex interaction of genetic and environmental risk factors. Studies point towards gene transcription, protein function, lipid metabolism and regulation of biliary lipid secretion in the formation of CGS.

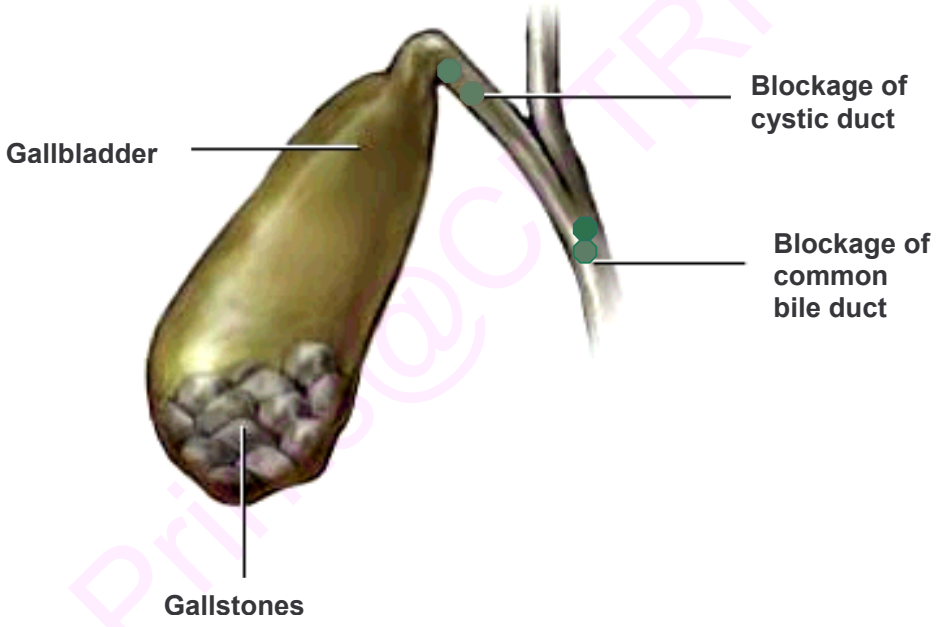

Fig 1.1: The diagram showing the gallstones in gallbladder and ducts connected to the common bile duct

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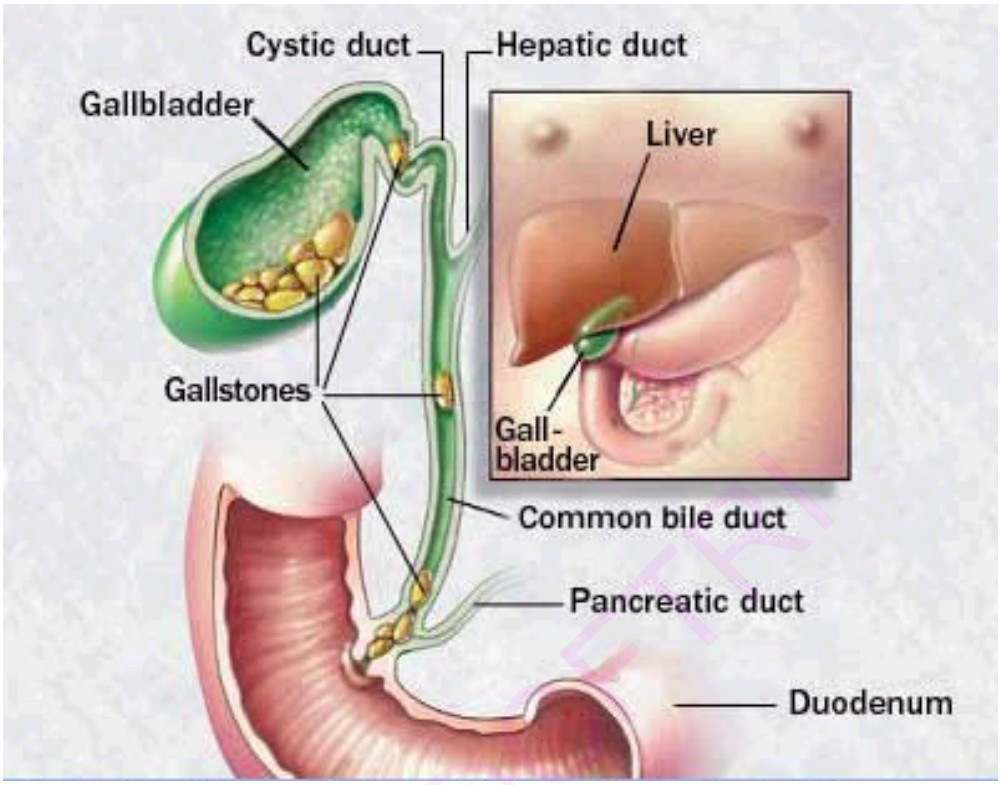

Fig 1.2: The diagram showing the gallstones in gallbladder connected with common bile duct

Source: Navigator.medschool.pitt.edu/34

Major biliary lipids

Cholesterol

Cholesterol is a sterol that is obtained in the diet or synthesized mainly in the liver. It is an amphipath, that is, it has hydrophilic (polar) and hydrophobic (non-polar) components. The molecule consists of three parts viz: a sterol nucleus and a side chain, both of which are hydrophobic, and a hydrophilic hydroxyl group. The latter is internalized, thus reducing its beneficial effect on aqueous solubility. As a result of its composition and structure cholesterol is very sparingly soluble in water, it has an aqueous solubility of 10^{-8} moles L^{-1} [Chijiiwa et al, 1988].

Phospholipids

The major phospholipid of bile is phosphatidyl choline (lecithin). Lecithin has backbone consisting of glycerol, linked with two fatty acids, phosphoric acid and choline. The choline group consists of hydrophilic portion of the molecule, the two

fatty acid chains are hydrophobic. Unlike the hydroxyl group of the cholesterol molecule, the hydrophilic group of lecithin is external and a very strong polar group. Spatially lecithin is a linear amphipathic molecule, with a hydrophilic head and a hydrophobic body and tail [Strasberg, 1998].

Bile salts

Bile salts are synthesized from cholesterol in the liver. In the course of synthesis, hydroxyl groups are added to the sterol ring of the cholesterol molecule and a carboxyl group to the side chain. The spatial orientation of these polar hydroxyl and carboxyl groups in most physiologic bile salts is such that they align along one side of the molecule. The bile salt molecule may therefore be thought of as a plate one side of which is hydrophilic and the other hydrophobic [Donovan and Carey, 1990].

The behavior in aqueous solution of these three amphipathic biliary lipids depends on their structure. At any concentration above its very low aqueous solubility, cholesterol self-aggregates into solid cholesterol monohydrate crystals. Phospholipids have a somewhat higher aqueous solubility than cholesterol. However, at relatively low concentrations, phsopholipids also associate out of solution. They form a molecular bilayer in which the hydrophobic ends of the molecules are turned inward away from the aqueous environment and the hydrophilic ends are turned out toward the aqueous environment. This familiar structure is the basis of cell membranes. These sheets of molecular bilayers will close naturally to form hollow spheres known as vesicles. Vesicles can be seen by specialized light microscope, such as phase contrast microscope, and are very easily seen by electron microscope. Although vesicles may be very small and may not aggregate with each other to form larger vesicles for some time, vesicles are not in solution but are a solid phase. They are highly deformable and therefore are often called “liquid crystals”.

Bile salts monomers are quite soluble in water. Their aqueous solubility, which is approximately 10^{-3} mol/L is much higher than that of cholesterol or phospholipid monomers. Above this concentration referred to as the critical micellar concentration (CMC), associative behavior begins and bile salts aggregate into simple micelles. The exact CMC varies depending on the type of bile salt and other conditions. Micelles are molecular associations of bile salts in which molecules are aligned to present the hydrophilic surface to the aqueous environment. The number of molecules per simple micelle is between 4 and 25. Micelles are much smaller than

vesicles and are too small to be seen by electron microscope. Unlike self-aggregates of cholesterol or phospholipid, micelles are dispersed completely in solution [Strasberg, 1998].

Packing of biliary lipids

Any of the three biliary lipids may associate with each other. Phospholipid molecules may be incorporated into simple bile salt micelles to form mixed micelles. These molecular complexes are considerably larger than simple bile salt micelles. In an environment in which an excess of bile salts are present, phospholipid vesicles will be totally incorporated into mixed micelles. Cholesterol is readily incorporated into the phospholipid bilayer of vesicles and there becomes associated with the highly hydrophobic fatty acid chains. Similarly, cholesterol may be incorporated into micelles. Its solubility in simple micelles is low but mixed micelles have a much greater capacity to solubilize cholesterol because of the high affinity of cholesterol for the fatty acid chains of phospholipids. The incorporation of cholesterol into vesicles and micelles greatly enhances its transport in aqueous solutions such as bile [Strasberg, 1998].

The role of liver, gallbladder and intestine and the enterohepatic circulation in cholesterol gallstone disease is depicted in Fig.1.3. It also shows biliary lipid secetion.

Phase equilibrium, and the equilibrium phase diagram

The forms in which cholesterol can exist in bile is referred to as a “phase”. Thus the phases of the cholesterol are the monomeric phase (solid cholesterol crystals) and the vesicular phase in which cholesterol is incorporated into phospholipid vesicles, the micellar phase in which cholesterol is incorporated into mixed micelles.

Movement of cholesterol molecules between phases is possible and is governed by the energetics of the system. For instance, cholesterol monomers may move into the vesicular phase or the micellar phase. Cholesterol molecules may move out of a supersaturated vesicular phase to form cholesterol crystals. However, eventually all movement stops and a state of equilibrium is reached. Understanding the equilibrium is key to comprehending the physicochemical changes that lead to gallstone formation. Equilibrium is a state in which all acting influences are nullified by others resulting in a stable, balanced, or unchanging system [Donovan and

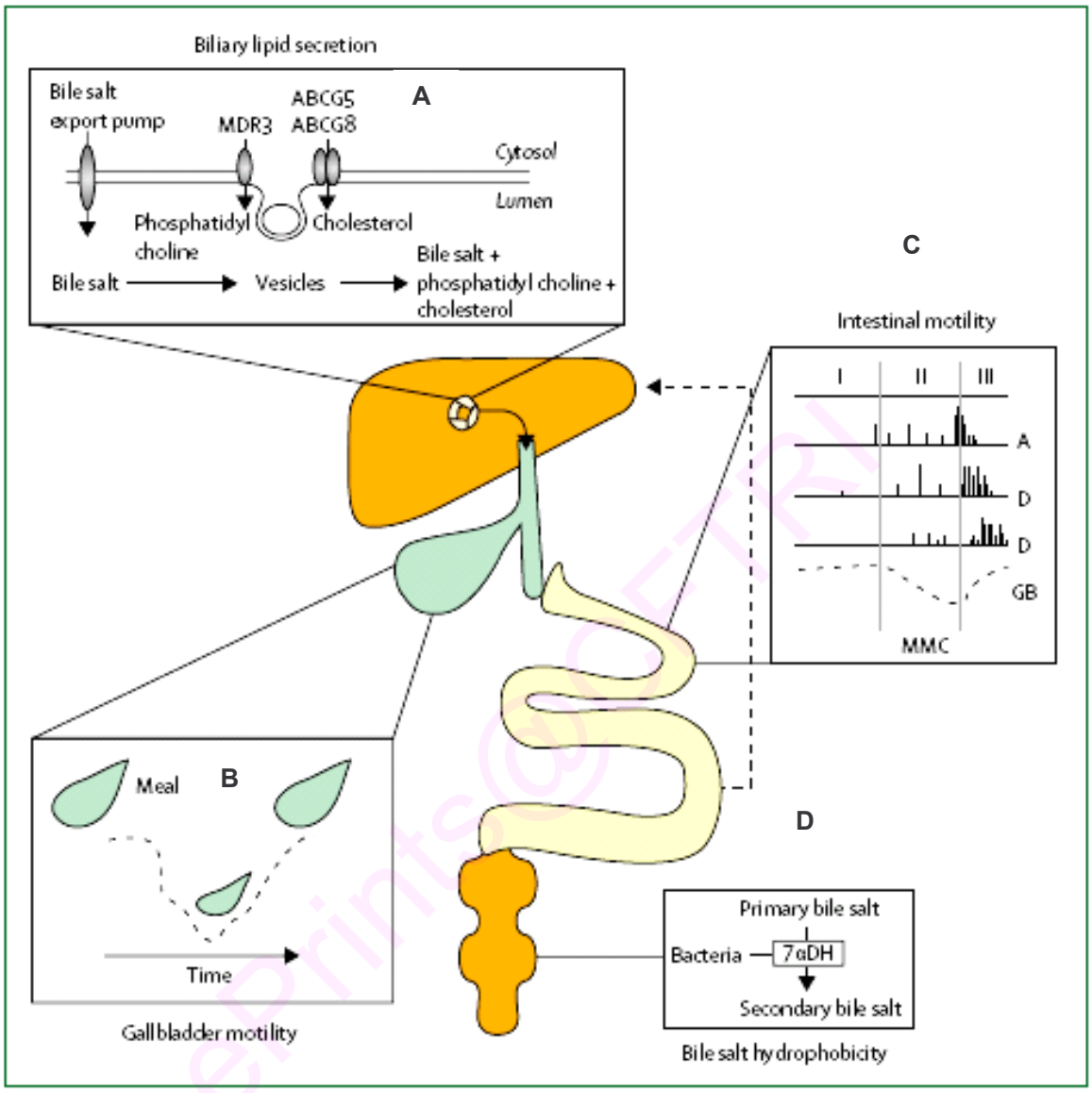

Fig 1.3: Role of the liver, gallbladder, intestine and the entero-hepatic circulation in cholesterol gallstone disease

The four boxes represent key physiological events in CGS prevention. Box A shows the pathways of biliary lipid secretion and nascent bile formation in the liver. Box B shows a schematic normal time dependent changes in gallbladder volumes before and after a meal. Box C shows the rhythmic cycles of gastric and small intestinal motility synchronized with periodic fluctuation of fasting gallbladder volume. Box D represents the hydrophobic secondary bile salts (deoxycholate and lithocholate) produced in the colon by bacterial 7∝-dehydroxylation of the primary bile salts (cholate and deoxycholate respectively). The broken line represents the enterohepatic circulation of bile salts.

Source: Portincasa, P; Moschetta, A and Palasciano, G (2006) Lancet , **368,** 230

Carey, 1990]. The equilibrium phase diagram predicts the phases present at equilibrium (Fig 1.4). The monomeric phase is not depicted in this diagram. By definition, a solution is supersaturated with respect to cholesterol when a solid phase is present at equilibrium – either vesicles or solid cholesterol monohydrate crystals or both. Unsaturated solutions contain only monomers and micelles. Although aqueous solutions may be prepared which are supersaturated on the basis that vesicles alone are present at equilibrium, the range of bile composition in humans is such that bile is either unsaturated or if supersaturated has cholesterol crystals with or without vesicles. Measuring the concentration of lipids in bile and plotting its relative composition on the phase diagram determines whether bile is supersaturated with cholesterol or not. Because this is cumbersome, the information on the phase diagram was mathematically converted to a cholesterol saturation index. If bile has a cholesterol saturation index greater than 1.0, it is saturated with cholesterol and will contain cholesterol crystals when that bile comes to equilibrium.

It is of critical importance in understanding the pathogenesis of cholesterol gallstones to be aware that bile is not secreted in a state of equilibrium. At the time of secretion, there are no cholesterol crystals in bile. Some supersaturated biles reach equilibrium and contain cholesterol crystals, which still in the biliary tree, but others do not. Also, some cholesterol crystals form in supersaturated bile in the biliary tree depends on certain kinetic factors.

Cholesterol saturation index

Cholesterol saturation index (CSI) or lithogenic index is a measure of supersaturation of bile with cholesterol. CSI is defined as –

$$CSI = \frac{\text{The actual amount of cholesterol in the bile}}{\text{Max. amount of cholesterol that can be solubilised in micelles}}$$

Bile is considered supersaturated with cholesterol if CSI is greater than 1 and unsaturated when CSI is less than 1.

Hydrophobicity index

Hydrophobicity index (HI) is the derivation of bile salt monomeric hydrophobicity index that quantitatively defines the composite hydrophilic-hydrophobic balance of a mixture of bile salts. The index is based on the logarithms of bile salt capacity factors

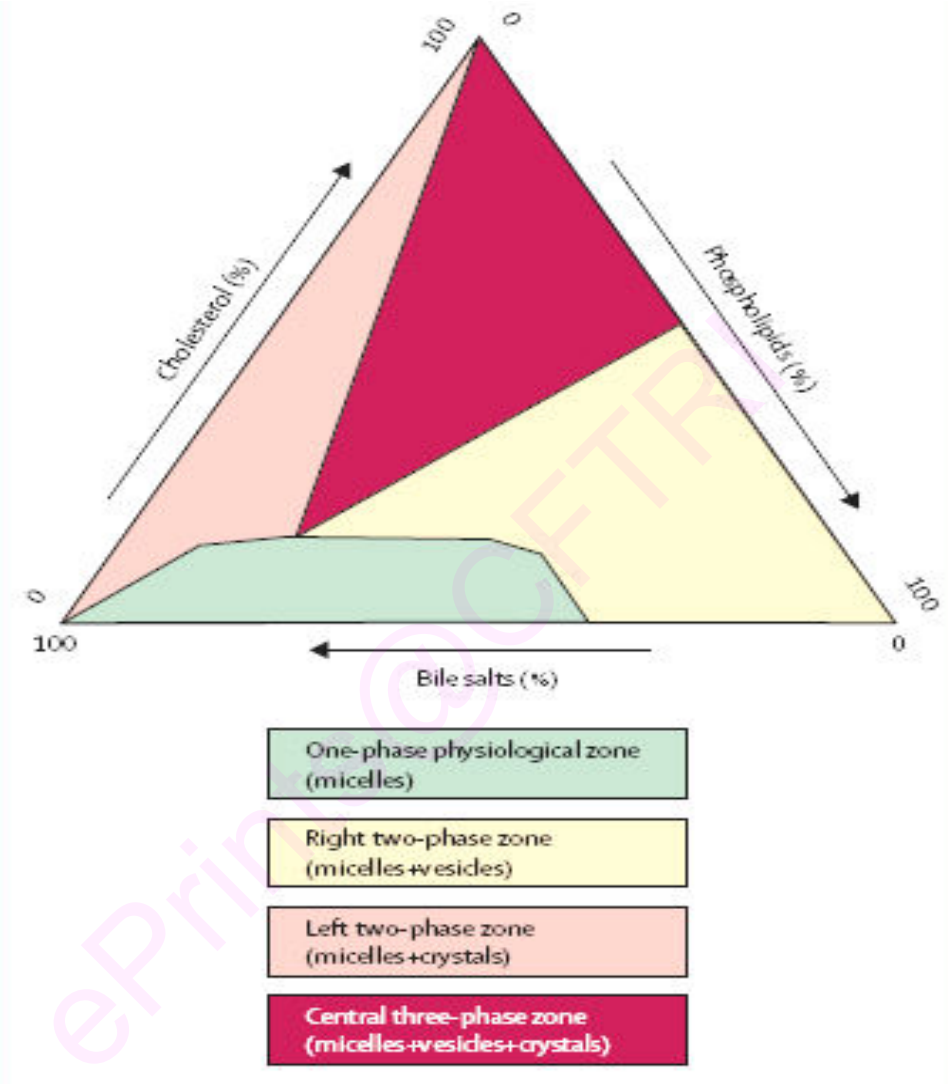

Fig 1.4: Schematic representation of the ternary bile salt-cholesterol-phospholipid phase diagram

Source: Portincasa, P; Moschetta, A and Palasciano, G (2006) Lancet , **368,** 230

to elute in the reversed phase HPLC (stationary phase: octadecyl silane; mobile phase: methanol – water; 70:30 w/w; ionic strength 0.15). The standardized arbitrary indices have been set as 0 to taurocholate and 1 to taurolithocholate. The indices of tauroursodeoxycholate, taurohyodeoxycholate, taurochenodeoxycholate and taurodeoxycholate were –0.47, -0.35, +0.46 and +0.59 respectively. Whereas for glycine conjugated bile salts these values are once again pH dependent. The average hydrophobic-hydrophilic balance of biliary bile salts is quantified by means of bile salt monomeric hydrophobicity index [Heuman, 1989].

Secretion and transport of cholesterol in bile

Hepatic cholesterol is derived either from preformed cholesterol taken up form the blood by the liver cell or from synthesis of cholesterol within the hepatocyte. Hepatic cholesterol may be exported into bile directly, used for bile salt synthesis, or converted into cholesterol esters. Bile salts are secreted directly into bile, and cholesterol esters are either exported from liver into serum or stored as such in the liver (Fig 1.5). Many of the enzymes and receptors involved in these steps are known. Certain of the risk factors associated with gallstone formation are known to affect the activity of these enzymes or expression of receptors governing these steps [Strasberg, 1998].

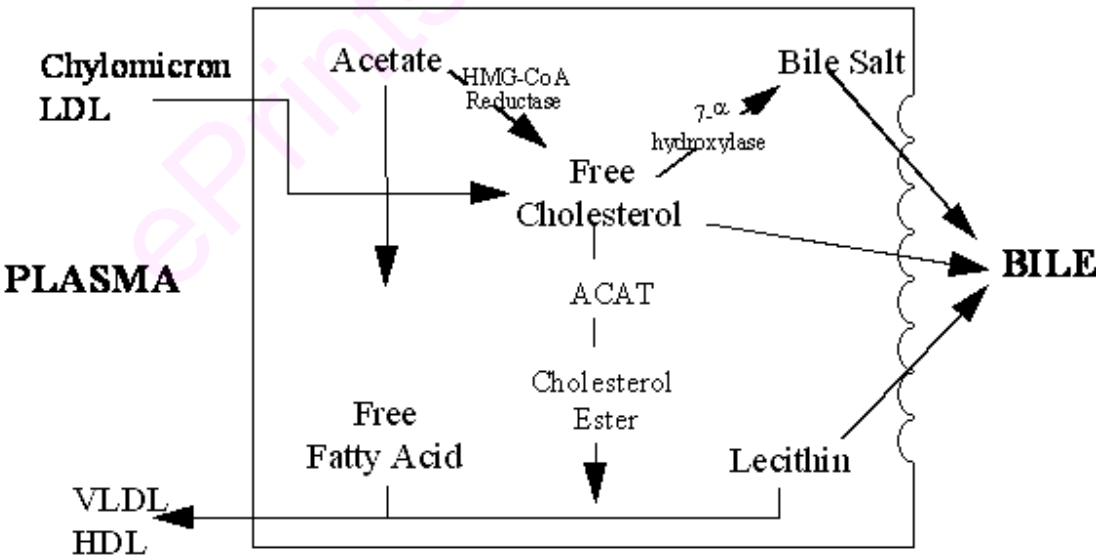

Fig 1.5: Source of biliary lipids

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Whether bile is supersaturated or not is largely determined at the moment it is secreted into the canaliculus, and reaches the gallbladder. Cholesterol is secreted into bile as cholesterol –phospholipid vesicles [Ulloa et al, 1987; Cohen et al, 1989]. Most of biliary cholesterol appears to be preformed; athough approximately 20% is synthesized. There is considerable evidence that bile salts are necessary for the "budding-off" of vesicles from the canalicular membrane, and this corresponds to the observation made years ago that bile salts stimulate the secretion of cholesterol and phospholipids into bile. Bile salts are probably secreted into the canaliculus in monomeric form and associate to form simple micelles.

The presence of vesicles and micelles in the same aqueous compartment provides the opportunity for lipid exchange to occur between cholesterol carriers, alluded to previously, with reference to the process by which vesicles are altered in the presence of micelles as vesicular "maturation". It is likely that maturation occurs as a result of direct contact between cholesterol carriers as well as movement of cholesterol monomers between carriers. The net effect is incorporation of vesicular lipid into simple micelles to make mixed micelles. In unsaturated bile, there is micellar excess and eventually all vesicular lipid becomes incorporated into micelles. There seems to be a tendency for vesicular phospholipids to be incorporated into micelles more readily than vesicular cholesterol. As a result, during maturation, vesicles become enriched with cholesterol. When the cholesterol-to-phospholipid ratio of vesicles exceeds 1.0, vesicles tend to become unstable and nucleate cholesterol crystals. In unsaturated bile, cholesterol enrichment of vesicles is of no consequence since eventually all vesicular lipid is incorporated into micelles [Hardison and Apter, 1972].

Pathway of crystal formation in supersaturated bile

Secretion of supersaturated bile is similar to that of unsaturated bile, the major difference being that the relative amount of cholesterol secreted into bile is greater. In the unsaturated bile, the difference being that residual vesicles still are present at the point that micelles have taken up cholesterol and phospholipids to capacity. For reasons already mentioned, such vesicles are enriched in cholesterol. Furthermore, these vesicles develop areas on their surface that are particularly enriched in cholesterol. The subsequent steps resulting in the appearance of cholesterol monohydrate crystals consist of vesicular aggregation and fusion, nucleation and crystal growth. Nucleation of cholesterol crystals takes place by vesicular

aggregation and fusion in supersaturated bile and was first shown by Halpern et al [1986 a, b]. Aggregation and fusion are events that lead to bring cholesterol-enriched zones on the vesicular surface into apposition, greatly facilitating nucleation of cholesterol monohydrate crystals. Initially crystals that have nucleated are very small and unstable, but with crystal growth they attain a stable size and eventually a size at which they are microscopically detectable. Sometimes the initial crystal is the classical cholesterol monohydrate crystal, but at other times the initial crystals are filaments, coils, or tubes that subsequently are transformed into the classical cholesterol monohydrate crystals [Konikoff et al, 1992] (Fig 1.6).

Stages of gallstone formation (Fig 1.7)

1. Supersaturation

Supersaturation is due to excessive cholesterol secretion, and there are many mechanisms responsible for this. Bile could theoretically become supersaturated as a result of excessive cholesterol secretion or reduced secretion of cholesterol carriers – bile salts or phospholipids. Formerly it was thought that reduced bile salts secretion was an important mechanism. Indeed, thin patients do have smaller bile salt pools and their bile salt secretion rates may be low, possibly because of inappropriately low bile salt synthesis rates. However, it now seems more likely that the small bile salt pool is secondary to stone formation. The enterohepatic circulation of bile salts is more rapid in these patients, increasing the return of bile salts to the liver, which secondarily reduces synthesis rates producing a small pool size. This leads to a new steady state, in which there is a smaller pool and more rapid cycling, but normal secretion rates and eventually normal synthesis rates. Therefore, although theoretically events other than cholesterol hypersecretion could induce cholesterol saturation, in reality, supersaturation usually appears to be due to cholesterol hypersecretion. However, there are multiple mechanisms producing cholesterol hypersecretion and many of these have been shown to be related to known risk factors for cholesterol stone formation. Some of the risk factors involved are :

Age

Supersaturation of bile increases with age [Valdivieso et al, 1978] because of increased choleseterol secretion [Einarsson et al, 1985]. Bile salt secretion is not

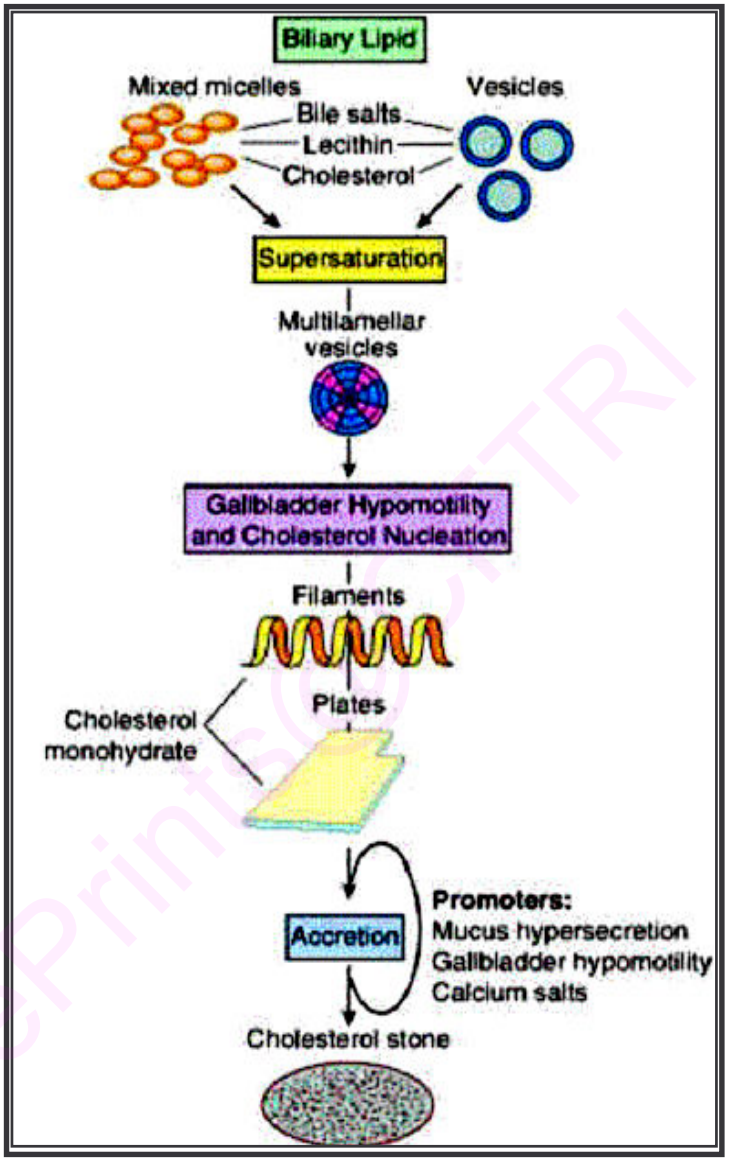

Fig 1.6: Stages of cholesterol gallstone formation

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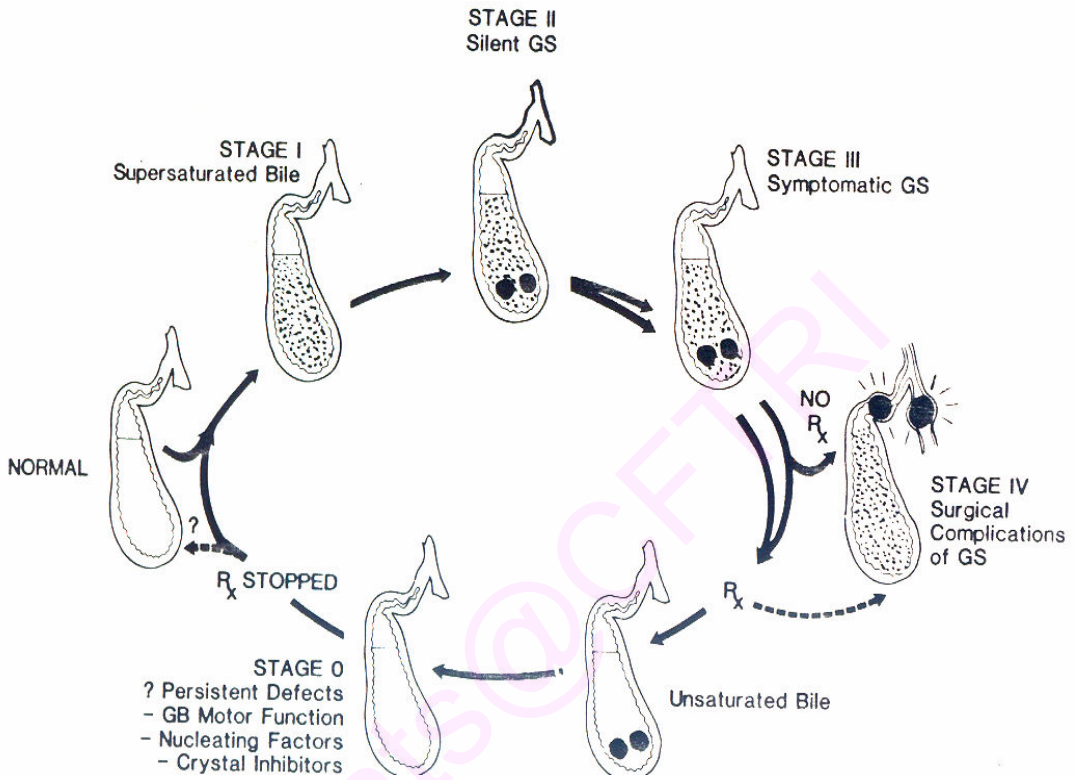


Fig: 1.7: Staging of cholesterol gallstones

Source: Dowling, R.H. and Paumgartner, G. Hepatology (1990) **12,** 234s

decreased, although the bile salt pool becomes smaller. The effect of age is independent of the influence of obesity, although the effect of these factors is additive. The cellular mechanisms responsible for increasing cholesterol secretion with age are not completely known. Recently it has been reported that cholesterol 7 ∝-hydroxylation, the rate limiting step in the bile salt synthesis from cholesterol, was significantly decreased in older patients compared to middle aged subjects [Bertocothi et al, 1993]. This suggests that increased cholesterol secretion in older subjects is linked to decreased utilization of hepatic cholesterol for bile salt synthesis.

Obesity

The linkage between cholesterol gallstones and obesity has been known for years. There is a linear correlation between body weight and cholesterol secretion into bile [Bennion and Grundy, 1975]. The main defect is excessive cholesterol synthesis and that neither reduced bile salt synthesis nor esterification of cholesterol contributes to the excessive cholesterol secretion. Liver of gallstone patients contains an intracellular vesicular fraction rich in lecithin. In obese subjects there is more rapid transport of this fraction into bile [Anderson, 1992].

Rapid weight loss by obese patients may contribute for gallstone formation since there is a sharp increase in cholesterol secretion into bile. Altered gallbladder motility and accelerated crystallization rates may also contribute to gallstone formation during rapid weight loss [Bennion et al, 1980].

Sex and pregnancy

Females have a strong risk factor for supersaturation of bile. Estrogen promotes secretion of cholesterol into bile [Everson et al, 1991]. Premarin has this effect as a result of enhancing hepatic lipoprotein uptake and inhibiting bile salt synthesis [Kern et al, 1981]. During the last two trimesters of pregnancy, cholesterol secretion into bile increases relative to bile salt and phospholipids secretion with the result that the cholesterol saturation index rises [Reichen et al, 1987]. Studies in hamsters also showed increased cholesterol synthesis during pregnancy [Braverman et al, 1980]. Decreased percentage of chenodeoxycholic acid in the bile salt pool during pregnancy also contributes to the excessive cholesterol synthesis which is otherwise an inhibitor of cholesterol synthesis [Everson et al, 1982]. Pregnant women and those taking contraceptive steroids have a slower rate of gallbladder emptying [Everson et al, 1982: Lawson et al, 1985] and prolonged gastrointestinal transit time [Kern, 1994].

Diet

Diet plays an important role in cholesterol supersaturation. General perception is that vegetarians do not form cholesterol gallstones. Cholesterol gallstones are common in populations eating a western diet, which is relatively high in animal fat. The incidence of cholesterol gallstones rises in populations shifting to higher consumption of fat in the diet. However relationship between diet and gallstones is complex [Khanuja et al, 1995].

2. Genetic mechanisms

Genetic mechanisms involved in gallstone formation have received little attention. In an important series of studies, it was demonstrated that there is a large inter-strain variability in gallstone formation in cholesterol fed mice [Pomare and Heaton, 1973]. Genetic analysis demonstrated that susceptibility to gallstone formation was a dominant trait determined by at least two genes. One, a major gene named Lith I, is mapped to mouse chromosome 2. Susceptible strains failed to down regulate cholesterol synthesis during cholesterol feeding. Although the mechanism is unlike the response of humans with cholesterol gallstones, the approach used in these studies shows promise for application in humans with gallstone diseases [Pomare and Heaton, 1973]. Lith genes in mice have been shown to determine biliary cholesterol supersaturation, mucin gel accumulation, gallbladder size, phase separation and prevalence of cholesterol gallstones [Wang et al, 1997]. Lammert et al, [1999] have showed that primary Lith gene phenotype alters the multiple hepatic lipid regulatory enzymes and induces secondary events to increase the availability of cholesterol to supply the sterol to the hepatocyte canalicular membrane for hypersecretion into bile. They have also shown that lith genes determine increased output of all biliary lipids with increased cholesterol hypersecretion disproportionate to lecithin and bile salt outputs thereby inducing lithogenic bile formation [Wang et al, 1999].

Deoxycholate enrichment of the bile salt pool

Enrichment of secondary bile salt deoxycholate in the bile salt pool is likely to induce the supersaturation of bile. Subjects with stones often have higher deoxycholate levels in bile [Di Donasto et al, 1986]. Feeding low doses of deoxycholate results in increased cholesterol saturation of bile [Berr et al, 1996] presumably deoxycholate is more efficient in stimulating cholesterol secretion by the liver than other bile salts. But the fact remains that enrichment of deoxycholate is much debated but for that

reason it is not possible to rule out this mechanism by which bile may get supersaturated.

Colectomy

Since too much conversion of cholate to deoxycholate results in supersaturated bile, it is paradoxical that colectomy, a procedure that results in virtual obliteration of the deoxycholate component of the bile salt pool also results in supersaturation. In the patients with ulcerative colitis a rapid increase in the CSI and appearance of cholesterol crystals in bile was noticed [Harvey et al, 1991a].

3. Accelerated crystallization

The second stage of stone formation is crystallization of cholesterol from supersaturated bile. It was through the application of physical chemistry to the clinical problem of cholesterol cholelithiasis. Initially it was suggested that gallbladder bile from patients with gallstones was supersaturated and control bile was not [Admirand and Small, 1968]. However, it rapidly became clear that many normal persons without cholesterol gallstones also have supersaturated bile [Holzbach et al, 1973; Holan et al, 1979a: Gollish et al, 1983]. Virtually all patients with cholesterol gallstones have supersaturated bile but reverse is not true. There are many stone-free persons with supersaturated bile than patients with supersaturated bile and cholesterol gallstones. In other words, supersaturation is needed for stone formation but supersaturation does not guarantee stone formation. It should be noted in this context that in Japan it appears that patients who form stones have highly supersaturated bile whereas persons without stones have unsaturated biles. But it is not the same with Canadian population. This variation might be due to some genetic reasons [Sanbaria, et al 1995].

Cholesterol crystallization and the gallbladder motility defect

The possible role of gallbladder motility on CGS formation was not established until shown by Everson et al, [1982] that during pregnancy gallbladder emptying was reduced and residual volumes were increased. Then few others have shown that some patients with cholesterol stones have a motility defect, and others have confirmed this. Subsequently it was found that obesity also exerts an effect on stone formation via impairment of gallbladder motility. Confirmatory evidence comes from animal models in which it was shown that the motility defect precedes the crystal formation [Fridhandler et al, 1983].

Cholesterol crystallization and the kinetic or "Nucleation" defect

The technique for measuring the rapidity of crystal formation from bile initially cleared of all detectable crystals by ultracentrifugation is termed as "nucleation time". But now more appropriately referred to as "crystal detection time" is used to establish the shortest possible time for the appearance of the detectable crystals [Holan et al, 1979b].

The kinetic defect accelerating crystallization must act by influencing one of the elements in the crystallization pathway leading to equilibrium (vesicle maturation, aggregation, fusion, nucleation, and crystal growth). It seems that the kinetic defect was impairment of a normal mechanism that retarded the progress towards equilibrium or the introduction of an abnormal mechanism that accelerated the process or both. Substances that potentially influence these mechanisms have been referred to as antinucleating or pronucleating factors. These terms are no more appropriate than "nucleating time", since such factors might act at any of the multiple steps leading to equilibrium and not just at the nucleation step. These are better referred to as procrystallizing and anticrystallizing factors [Sedaghat and Grundy, 1980].

Procrystallizing factors

Many compounds present in the bile have pronucleating effects. Mucus glycoprotein, calcium ions, bilirubin and low molecular weight proteins have been shown to have pronucleating effects [Gallinger et al, 1986: Harvey et al, 1986]. The 130 KD glycoprotein has been isolated from T-tube biles of CGS patients which is however found in normal subjects but its content was increased in the cholelithiasis prevailing conditions. This glycoprotein was not found in gallbladder mucosal homogenates but has been previously isolated from T-tube bile and hepatic bile and it was speculated that this glycoprotein is produced in liver or bile duct epithelium. Con-A binding glycoprotein from bile shows the pronuleating activity as demonstrated by Groen et al [1990]. This nucleating glycoprotein lost its property when treated with mixed glycosidases suggesting that the carbohydrate residues of these glycoproteins are important for pronucleating effect and extremely small quantities of glycoproteins are enough to cause cholesterol crystal nucleation in model bile [Groen et al, 1988: Lee and Smith, 1989]. Immunoglobulins also have been identified as pronucleating factors [Harvey et al, 1991b].

Mucin, a glycoprotein of gallbladder mucus, found in animals is a potent pronucleator [Smith, 1987]. In cholesterol cholelithiasis, mucin hypersecretion precedes nucleation of cholesterol crystal and CGS formation [Lee et al, 1981]. In animal studies using ground squirrels or prairie dogs fed a lithogenic diet it has been shown that mucus hypersecretion precedes CGS formation. Moreover mucin promotes cholesterol crystal nucleation in model bile.

Nucleation of cholesterol crystal from lithogenic biles can however occur in the absence of mucin. It has been demonstrated that fibronectin, a disulfide bonded dimer of molecular weight 440 KD present in bile, shortens the nucleation time of both native and model biles [Chijiwa et al, 1991].

Calcium plays a major role in the precipitation of cholesterol crystals. It promotes cholesterol crystal growth by (a) decreasing the solubility of mucin, (b) promoting the fusion of vesicles and hence nucleation of cholesterol crystals and (c) acts as a nidus for the precipitation of cholesterol from supersaturated bile [Morre, 1990].

Anticrystallizing factors

Parallel to the procrystallizing factors, many anticrystallizing factors in the bile are also present. The apolipoprotein A_1 a 120 KD glycoprotein [Kibe et al, 1984] and a 15 KD protein [Ohya et al, 1993] has anticrystallizing effect. Busch et al, [1995] have isolated several anticrystallizing proteins that appear to bind to cholesterol crystals, suggesting that they are crystal growth inhibitors of which 63 KD novel protein, isolated by lectin chromatography is important. Previously from our laboratory anticrystallizing factors induced in rat bile by feeding capsaicin and curcumin was reported [Hussain and Chandrasekara 1994a]. The stimulation of production of anticrystallizing factors by dietary additives or intervention is potentially a means of preventing cholesterol gallstones.

Stone formation from crystals

Sludge, sediment, and stone formation

"Sludge" the term used in the 1970's in the liver transplantation literature to refer to necrotic collagen that obstructed bile ducts [McMaster et al, 1978]. Sludge also refers to material that precipitates in bile ducts in association with cholangiohepatitis or stepts [Chen et al, 1988]. This was also used simultaneously to describe echogenic material that layered out in the gallbladder in some patients who had been fasting. This is now a commonly used term. But in this context, it should be

remembered that it is a contraction of "echogenic gallbladder sludge", i.e; the material detectable in the gallbladder on ultrasonography.

Sediment refers to solid material detectable by microscopy. Bile normally contains sediment but it is sparse and consists of mainly dead cells. Pathologic sediment contains microcrystals of cholesterol or calcium bilirubinate or both. Pathologic sediment is usually not detectable by ultrasonography, but if sludge is detected there will always be pathologic sediment. Sludge is composed of bilirubinate microcrystals and mucus and in some cases cholesterol crystals are also present. This is not surprising since the conditions leading to sludge formation such as fasting lead to concentration of bile, one of the mechanisms by which crystallization is accelerated.

The Wolpers model of cholesterol stone formation-

Wolpers and Hofmann [1993] delineated the scheme for the formation of cholesterol gallstones. They used cholecystography, macroscopic examination, radiography, scanning elecrtron microscopy, and chemical analysis of gallstones. Solitary gallstones form from free-floating crystal laminae of cholesterol. These laminae aggregate loosely and undergo external compaction and internal remodeling by movement of cholesterol molecules to form compact spheroids. Multiple cholesterol gallstones form without a precursor phase. Innumerable very thin cholesterol crystals appear that abruptly aggregate to form spheres of up to 1mm in diameter. A second aggregation takes place within 3 months in which these spheres coalesce to form mulberry stones. Mulberry stones are transformed into either faceted stones or barrel stones over a period of 3 years. The smallest spheroids are termed, as "lithons" contained no calcium bilirubinate. Mulberry stones appear to be aggregations of lithons. Sanabria et al, [1994, 1996] demonstrated that cholesterol gallstones were porous to large molecules and provided evidence that pigmentation was secondary.

To summarize many advances in understanding gallstone formation is explored and being explored. The keys to stone formation are supersaturation and crystallization, both of which are influenced by many variables. In some cases it appears that deoxycholate excess as well as supersaturation are needed to trigger inflammation in the gallbladder. But major step has been the supersaturation of bile and gallbladder inflammation and secretion of procrystallizing agents.

Symptoms of CGS pathogenesis

Gallstones are so common they are blamed for many digestive symptoms but they are probably innocent in most cases. This concept is particularly important because ultrasonography is widely used in investigating patients and asymptomatic gallstones are often an incidental finding.

The symptoms of gallstones include acute cholecystitis (a febrile illness with pain and tenderness in the right upper quadrant), biliary colic, jaundice (often painful) and acute pancreatitis. Biliary colic is over diagnosed. It consists of clear-cut, well-remembered attacks of severe upper abdominal pain lasting at least half an hour. These together are termed as "Murphy's syndrome" [Malcom and Bateson, 1990].

Diagnosis

Laboratory tests include a complete blood count, liver function tests, and serum amylase and lipase levels. Ultrasonography and cholescintigraphy are the imaging studies most helpful and often used in the diagnosis of gallstone disease. Definite gallstones are seen as sono-dense mobile areas within the gallbladder and throw an acoustic shadow. It can also allow measurement of the diameter of the common bile duct and show the liver and hepatic bile ducts. Computed tomography is not as accurate as ultrasonography in detecting gallstones and is therefore not a good screening study in evaluating patients with probable chronic biliary disease. Endoscopic retrograde cholangiopancreatography (ERCP) is currently the only reliable and widely available investigation for duct stones. In future, magnetic resonance cholangiography may replace ERCP for diagnostic purposes [Vogt, 2003].

Treatment

Depending on the nature of the gallstones, various approaches are taken to cure them, which include –

Lithotripsy

It is the method of breaking the gallstones by extracorporeal shock waves [Sackmann et al, 1988] practiced in some of the highly specialized centres, but this treatment often leaves fragments that have to be cleared by subsequent bile acid therapy. About 1/3 of patients will experience colic, a direct result of external shock wave lithotripsy. But the draw back of this is that only 30% of the CGS patients are eligible and the stone reoccurrence chances are very likely.

Cholecystectomy

It is the surgical procedure, which is practiced from very early days. The introduction of laparoscopic cholecystectomy over the past 10 years has changed the surgical practice greatly. It is effective and safe but involves complete removal of gallbladder. The mortality rates are very less (0.17%) particularly if performed electively in patients younger than 65 years. Limitations are: aged patients, other complications such as liver cirrhosis, severe diabetes and of course patients mindset for not loosing an organ!

Cholelytholytic drugs

Bile acid therapy is an attractively safe option but is suitable for only few patients and is unsuitable in those with severe recurrent symptoms. The best bile acid therapy currently available is probably ursodeoxycholic acid (750 mg/day) or combination of the former with chenodeoxycholic acid will be effective. Ursodeoxycholic acid increases bile acid production whereas, chenodeoxycholic acid inhibits cholesterol synthesis and secretion. Recurrence of stones after treatment can be expected within 5 years in about 40% of patients [Hood et al, 1993].

Alternative treatment

"Gallbladder flush" or "Liver flush" is a popular remedy in alternative medicine for gallstone treatment. In this, the patient is made to drink 4 glasses of apple cider and eat 5 apples/day for 5 days and followed by brief fasting and magnesium is administered. Now the patient is given large quantity of lemon or grape juice mixed with olive oil or oil before bed. In the next morning the patients pass number of green or brown pebbles purported to be stones flushed from the biliary system. But this treatment has its own limitations and complete cure is not achieved by this treatment [Savage et al, 1992].

Dietary intervention

Dietary intervention can be an alternative approach? The answer is "YES". Dietary intake has long been looked upon as a potentially modifiable risk factor for gallstone disease. Studies show the epidemiological evidence for an association between dietary intake and gallstone disease. The most of the attention has been received by six dietary components viz: energy intake, fatty acids, cholesterol, carbohydrates, fibre and alcohol [Tseng et al, 1999].

Energy intake

Excessive energy intake is one of the risk factors for gallstone pathogenesis. Obesity increases the risk for gallstones by contributing to an elevated flux of cholesterol from the liver. Obesity has been associated with increased cholesterol synthesis possible in part through its association with hyper-insulinaemia, as well as increased biliary cholesterol secretion and cholesterol supersaturation of the bile [Hayes et al, 1992; Heaton, 1984].

Fatty acids

Dietary fatty acids play a role as a risk factor for gallstone. Several mechanisms specific to different types of fat have been proposed. But changing either the type or the amount of fat in the diet in human feeding studies has not produced any consistent effect on bile cholesterol content or on gallstone occurrence. High fat overall intake may also increase the risk of gallstones by contributing to obesity [Hayes et al, 1992].

Cholesterol

Increased cholesterol intake has been one of the major risk factors that contribute to bile cholesterol saturation. However, results of human feeding studies have been inconsistent. As far as high cholesterol foods are concerned no positive association was found [Den Besten et al, 1973].

Highly refined carbohydrates and low fibre

Highly refined carbohydrates coupled with low fibre intakes may increase the risk of gallstone development. Insoluble fibre may protect against gallstone occurrence by speeding intestinal transit time and reducing the generation of secondary bile acids such as deoxycholate [Marcus and Wheaton, 1986 (a), (b)] which has been associated with increased cholesterol saturation of the bile [Hayes et al, 1992].

Alcohol

Moderate alcohol intake may protect against gallstone development possibly through its association with reduced biliary cholesterol saturation and higher serum HDL [Thornton et al, 1983a; Lavecchia et al, 1994].

Other foods and food patterns

Vegetarians may be at decreased risk of gallstone disease. Vegetables, vegetable oil, vegetable protein or crude fibre from vegetables have been reported to be

beneficial in overcoming gallstone disease [Pixley et al, 1985]. Other specific foods of interest like fruits, beans and pulses and caffeine showed to have inconsistent effect on gallstone disease [Kratzer et al, 1997].

Spices

Spices such as turmeric, red pepper, black pepper, ginger, onion, garlic, cumin, coriander, fenugreek etc. are being used for thousands of years in the Middle East and tropical countries due to their organoleptic and health beneficial properties. The spice ingredients impart characteristic flavor, aroma or piquancy and color to foods. In addition to their ability to increase the palatability of food, they have been long recognized to possess medicinal properties and thus found pharmacological applications. Recently Srinivasan et al, [2004] reviewed the available literature related to "Spices as beneficial hypolipidemic food adjuncts". It has been shown that spices like fenugreek, garlic, ginger,onion, red pepper and turmeric were effective as hypocholesterolemic agents under experimentally induced hypercholesterolemic and hyperlipidemic conditions. Some of them like garlic, onion, fenugreek etc; are effective even in humans with lipemia. The beneficial effects on lipid metabolism of these spices would probably be in the order: garlic > onion > red pepper/ capsaicin> turmeric/curcumin > fenugreek > ginger.

Spices have long been recognized for their digestive stimulant action. It has been well established that they may influence salivary, gastric, biliary and pancreatic secretions. In this context the digestive stimulant action of spices is probably exerted through stimulation of the liver to produce and secrete bile rich in bile acids, which play a very important role in fat digestion and absorption [Platel and Srinivasan, 2004]. The hypocholesterolemic spices curcumin, capsaicin, (the active principles of turmeric, red pepper respectively), ginger and fenugreek stimulated bile acid production by the liver and its secretion into bile [Bhat et al, 1984,1985; Bhat and Chandrasekara, 1987]. Ramprasad and Sirsi [1956] observed the choleretic effect of curcumin (sodium salt) in anaesthetized dogs. Curcumin almost doubled the bile production with associated increase in bile salts, bilirubin and cholesterol. Though cardamom is not a hypocholesterolemic spice, the cholagoguic action of its active ingredient (glucosides of terpeneol) increased bile acid output in rats [Yamahara et al, 1985]. Among the various spices studied for influence of bile acid output, fenugreek had the highest stimulatory influence on bile acid secreton among the various spices with increase of 80% over the control, followed by cumin (71%),

curcumin (62%), coriander (59%), tamarind (58%), mustard (50%), onion (47%) and ajowan (30%), capsaicin and mint (17%) [Bhat et al, 1984; Sambaiah and Srinivasan, 1991; Platel and Srinivasan, 2000]. The spices that did not showed a bile acid stimulatory action were; piperine, asafetida, fennel, cinnamon and garlic [Bhat and Chandrasekara, 1987; Sambaiah and Srinivasan, 1991; Platel and Srinivasan, 2000]. Apart from this, it was also showed that curcumin (0.5%) and capsaicin (0.05%) when fed with diet showed antilithogenic potential [Hussain and Chandrasekhara, 1994b].

Garlic and onion are widely used in the food preparations and have been shown to possess hypolipidemic and hypocholesterolemic activities in both experimental animals and humans [Kamanna and Chandrasekhara, 1982; Augusti and Mathew, 1973; Kleijnen et al, 1989]. Similarly, sesame seed is used as a health food since ages. Sesame oil is widely used in the Indian culinary is known to possess antioxidant and hypolipidemic effects. These features have been attributed to the presence of characteristic lignans such as sesamin, sesamol, episesamin and sesamolin in the sesame oil apart from their fatty acid composition. Sesamin is known to possess hypocholesterolemic property and it is brought about by increasing the excretion of cholesterol in bile and by inhibiting the cholesterol biosynthesis [Hirose et al, 1991].

Studies indicated, that garlic, onion and sesame oil possess hypocholesterolemic and hypolipidemic effects in experimental animals as well in humans by interfering at different steps in the lipid metabolism. On this basis, one can hypothesize that these spices and sesame oil could effectively interfere with the events leading to the formation of cholesterol gallstones, where supersaturation of bile with cholesterol is the pre-requisite for the onset of cholesterol gallstones. Hence garlic, onion and sesame oil could reduce, regress the formation or preformed cholesterol gallstones arising due to excessive cholesterol in bile.

Scope of the present investigation

An increased level of serum cholesterol is a major risk factor for diseases like cardiovascular disease (CVD), hypertension, obesity, gallstone pathogenesis, diabetes etc. Intensive clinical and experimental research has been carried out to understand the etiology of these diseases and for their treatment. Often sedentary life style, environmental factors and genetic predisposition play a pivotal role in the onset of these diseases. CVD like atherosclerosis remains often unnoticed till the end and proves fatal. Cholesterol gallstone (CGS) pathogenesis is not fatal but often results in severe gastroenterological disorders leading to several complications.

The treatment for CVD includes cholesterol synthesis inhibition drugs (statins), by-pass surgery at advanced stages, insertion of stents to clear the plaques in the blood vessels, etc, which is highly expensive and too risky for patients. On the other hand CGS is often unnoticed and is treated by oral drugs or lithotripsy. But often this may be not feasible in patients due to other ailments associated with CGS and patient is left with no other choice than cholecystectomy.

Fats and oils are essential component of diet. The quality of fat is determined by its fatty acid composition and unsaponifiable material, which plays a prominent role in keeping health. Often consumption of fat and quality of oil is a topic of debate. Hence sesame oil, which is used as health oil since time immemorable, was studied.

Spices - the natural esoteric food adjuncts have been in use for thousands of years are recognized for their several medicinal properties. Some of the commonly used spices/spice principles like turmeric (curcumin), red pepper (capsaicin), garlic, onion etc., exhibit hypocholesterolemic and cholagoguic activity. Food is subjected to processes like cooking, boiling, frying etc. before consumption which alters the physical and chemical characteristics of diet. Hence heat processing on food components such as garlic and onion is needed.

Eventhough hypolipidemic and antioxidant effects of sesame lignans were studied, delineation of the beneficial effects of whole sesame oil use is lacking. In the present study the hypocholesterolemic and antioxidant property of the SO was studied in experimental rats. Further, SO, garlic and onion (raw and heat processed) were studied in detail for their efficacy in reducing or inhibiting CGS formation. The rationale for selection of these was SO, garlic and onion were hypocholesterolemic in nature and cholesterol reducing food components may be promising in overcoming formation and regression of CGS. The CGS induction and regression was studied in

mice. The CGS were graded, cholesterol saturation index (CSI) and hydrophobicity index (HI) in bile were calculated. The serum, liver and biliary cholesterol levels were also determined. The cholesterol metabolizing enzymes like HMG-CoA reductase, cholesterol 7α-hydroxylase and sterol 25-hydroxylase activities were studied during induction and regression of CGS.

The biliary proteins from hepatic bile of rats fed spices (garlic and onion) and SO were separated and studied for cholesterol crystal nucleation in supersaturated model bile. In order to understand the mechanism of cholesterol crystal nucleation, biliary cholesterol carriers, viz; vesicles and micelles were separated from the model bile by gel filtration and the influence of biliary protein on vesicle and micelles was also examined.

The specific objectives of the current investigation were as follows;

1. To study the effect of sesame oil on cholesterol metabolism in normal and hypercholesterlemic condition.
2. To study the antioxidant and antilithogenic potential of sesame oil.
3. To study the effect of raw and heat processed garlic, onion and sesame oil on cholesterol gallstone induction.
4. To study the effect of raw and heat processed garlic and onion on pre-established cholesterol gallstones.
5. To study the effect of bile from raw and heat processed garlic, onion and sesame oil fed animals on supersaturated model bile and cholesterol carriers in cholesterol gallstone formation.

Chapter – II

Materials and methods

Acrylamide, ascorbic acid, adenosine diphosphate, bovine serum albumin (BSA), bile acids (kit), bile salts (kit), boron trifluoride in methanol, cholesterol, 27-hydroxycholesterol, 7α-hydroxycholesterol, cholesterol oxidase, commassie brilliant blue, di-thio-bisnitrobenzoic acid (DTNB), dipalmitoyl phosphatidyl choline, digitonin, dithiothreitol, EDTA, HEPES, HMG-CoA, 3α-hydroxysteroid dehydrogenase, heparin, hydrazine hydrate, hydrogen peroxide, low molecular weight protein standard kit for electrophoresis, manganese chloride, NAD, NADH, NADPH, N,N-methylene-bis-acrylamide, sodium dodecyl sulphate (SDS), sodium metaperiodate, Triglyceride purifier, 1,1,3,3-tetraethoxypropane, N,N,N',N'-tetramethylethylenediamine (TEMED), thiobarbituric acid (TBA), tripalmitin, tris-HCl, urethane, xanthine, xanthine oxidase and Sephadex G-100 were purchased from M/s Sigma Chemical Co; St. Louis, MO, USA. Fatty acid standards were procured from M/s Nu Check Prep. Inc., Elysian, Minnesota, USA. Polyethylene tubing (PE-10) was purchased from M/s Thomas Scientific, New Jersey, USA. Con-A sepharose was obtained from M/s Bangalore Genei, Bangalore, India. DL-alanine, choline chloride, di-nitrophenyl hydrazine (DNPH), L-aspartate, α-cellulose, mercaptoethanol, methionine and sodium pyruvate were purchased from M/s Hi Media Laboratories, Mumbai, India. Casein was purchased from M/s Nimesh Corporation, Mumbai, India. All other chemicals used were of finest quality available and solvents were distilled before use.

Sugar, sesame oil (*Sesamum indicum L*) (SO) and refined groundnut oil (GNO) were purchased from the local market. Sunflower oil (SFO) was from Deparment of Protein Chemistry and Technology, of this institute. The dehydrated onion (*Allium cepa)* and garlic (*Allium sativum)* powder used in the experiment was a generous gift from M/s Indo Nissin Foods Ltd. Anekal; Bangalore, India.

Animals

Rats

Male wistar rats [OUT-Wistar, IND-cft (2c)] weighing about 30 g obtained from Animal house facility of this institute (Central Food Technological Research Institute, Mysore) were used. They were placed in individual cages in an approved animal house facility with 12 h light and dark cycles with temperature 25 ± 2°C and fed fresh diets daily. The animals had free access to food and water throughout the study. The food intake and growth of the animals were monitored at regular intervals.

Mice

Male swiss albino mice weighing about 22 g were used for cholesterol gallstone induction and regression studies. The animals were grouped on the basis of their body weight and housed in the polypropylene cages with saw - dust as bedding. Animals were fed *ad libitum* and had free access to water through out the experimental period.

All animal studies were executed according to accepted guidelines for the care and use of laboratory animals. The Institutional Animal Ethical Committee of Central Food Technological Research Institute, Mysore, approved animal experimental protocols.

Heat processing of onion and garlic

The garlic and onion powder were added to the boiling water and boiled for 15 minutes continuously with constant stirring as practiced normally in the Indian culinary and used in the experiments.

Diets: AIN-76 semi purified basal diet was fed to animals daily. Composition of the basal AIN – 76 diet used in experiment with rats and mice is given in table 2.1 and 2.2 respectively.

Fat used was GNO, SFO or SO for preparing different diets. Diets were prepared by mixing the ingredients in a mechanical mixer and pellets were prepared by using hand-operated pelletizer. Diets were stored at 4°C in air-tight containers.

Table 2.1: Composition of AIN-76 basal diet used in experiments with rats

Ingredients	% Composition
Sucrose	60
Casein	20
Cellulose	5
Choline chloride	0.2
Methionine	0.3
Mineral mix*	3.5
Vitamin mix*	1
Fat	10

*AIN-76 vitamin and mineral mix were used

Ref: Bieri, J.G., Stoewsand, G.S., Briggs, G.M., Phillips, R.W., Woodard, J.C. and Knapka, J.J. (1980) J. Nutr. **110**, 1726

Table 2.2: Composition of AIN-76 basal diet used in experiments with mice

Ingredients	% Composition
Sucrose	65
Casein	20
Cellulose	5
Choline chloride	0.2
Methionine	0.3
Mineral mix*	3.5
Vitamin mix*	1
Fat (GNO)	5

*AIN-76 vitamin and mineral mix were used

Ref: Bieri, J.G., Stoewsand, G.S., Briggs, G.M., Phillips, R.W., Woodard, J.C. and Knapka, J.J. (1980) J. Nutr. **110**, 1726.

The different test diets were prepared by adding raw or heat processed (boiling for 15' in water) garlic (0.6%) or onion (2%) to the AIN-76 basal diet. In sesame oil (SO) group GNO was replaced by sesame oil. The diets were made isocaloric by varying sucrose content.

Lithogenic diet (LG) was prepared by adding 0.5% cholesterol and 0.25% bile salt to the AIN-76 basal diet. The diets were made isocaloric by varying sucrose concentration.

Proximate analysis

Proximate analysis of the onion and garlic powder, which includes determination of moisture, fat, ash, protein [Oser, 1965], fibre [AOAC, 1995] and carbohydrate content in the samples was carried out using standard procedures [Raghuramulu et al, 2003].

Estimation of allicin content

The allicin content was determined by measuring the decrease in optical density (OD) at 412 nm after incubation of garlic and onion samples for 30 min with NTB at room temperature as described [Talia et al, 1998]. Briefly, samples were added to tubes containing1.0 mL of NTB ($1.2X10^{-4}$) in 50 mM sodium phosphate, 1mM EDTA at pH 7.2 and incubated for 30 min at room temperature. At the end of the incubation, decrease in optical density was measured at 412 nm and the content of allicin was calculated using its molar extinction coefficient of $2.83 \times 10^4 M^{-1}cm^{-1}$.

Table 2.3: Proximate composition of garlic and onion powder

Components (%)	Garlic	Onion
Moisture	11.50±1.08	4.75±0.39
Fat	0.625±0.08	0.87±0.11
Ash	3.10±0.72	3.50±0.97
Protein	23.10±2.31	19.66±2.13
Fibre	0.78±0.09	1.68±0.26
Carbohydrate	60.89±0.06	69.54±0.30
Allin*	210.30±16.32	25.44±2.32
Allicin (μmol/g)	7.09±1.31	2.82±0.37

Values are mean ± SD of 4 separate analysis
*As total pyruvate (μmol/g)

Estimation of sesamol and sesamin

The sesame oil was dissolved in 3 volumes of acetone and the unsaponifiables were extracted with acetone in cold. The unsaponifiable fraction was concentrated and subjected to HPLC for seperation of lignans. The lignans were separated using using Shimadzu Spherisorb-RP-18 HPLC column using methanol:water (70:30) solvent system at a flow rate of 0.8 mL/min and the lignans were detected using UV detector according to Amarowicz et al. [2001].

Isolation of serum and tissues

Animals were fasted overnight and sacrificed under ether anesthesia. Blood was drawn by cardiac puncture and kept at 4°C for 2 h. Serum was separated by centrifugation at 1100 X g in a table top (Remi 8 C) centrifuge for 20 min and serum was stored at –20°C till analysis. Liver and other tissues were removed and rinsed with ice-cold phosphate buffered saline (pH 7.4). The tissues were then blotted, weighed and stored at –20°C till analysis.

Isolation of gallbladder, CGS scoring and bile collection in mice

The gallbladders from mice were carefully removed from liver without puncturing, cleaned of extraneous tissue, blotted gently with tissue paper, weighed and placed individually on a glass plate with a number of concave wells. The glass plate was placed on brightly illuminated background and observed under a magnifying lens. Four people were evaluated the CGS. The grading of the stones was done on a four-point scale. The absence or presence of the gallstones was marked by "-" or "+" respectively. After CGS scoring, the gallbladders were punctured and the contents of 3 to 4 gallbladerrs were pooled and stored at –20°C till further analysis.

Bile cannulation in rats

Rats were anaesthetized with urethane (1.2 g/kg body weight) by intraperitonial injection. Laparatomy was performed and common bile duct was cannulated with polyethylene tubing (PE-10). Bile was collected for 3 h in sterile tubes during which the body temperature of the animals was maintained at 37°C using incandescent lamps. The volume of the collected bile was then measured and stored for further analysis at –20°C.

Extraction of serum and liver lipids

Total lipids from serum and liver were extracted by the method of Folch et al [1957]. Briefly, 1 g of liver homogenized with 1 mL of 0.74% potassium chloride solution in a Potter Elvehjem homogenizer. Twenty mL of chloroform: methanol (2:1, v/v) was added and homogenized for 2 min. The mixture was left overnight and filtered through a Whatman no.1 filter paper. Three mL of 0.74% potassium chloride was added and mixed well by vortexing. The solution was allowed to stand at room temperature. The upper aqueous layer was removed carefully and the lower phase was washed with 3 mL of chloroform: methanol: water (3:48:47, v/v) mixture each time and the upper layer discarded. Finally the lower layer made into single phase by addition of few drops of methanol and used for lipid analysis. Similarly 1 mL of serum was mixed with 20 mL of chloroform: methanol (2:1, v/v) and filtered through a Whatman no.1 filter paper and further proceeded as described earlier.

Bile analysis

Biliary lipids were extracted by the method of Bligh and Dyer [1959]. Bile (0.8 mL) was mixed with 1 mL of chloroform, 2 mL methanol, vortexed and kept for 30 min. One mL chloroform and 1 mL water were added and kept for 15 min and centrifuged. The upper methanolic layer was taken for bile acid and uronic acid analysis and lower chloroform layer for lipid estimation. Total bile acids in methnolic layer were estimated by using 3 $\propto$- hydroxysteroid dehydrogenase [Turley and Dietschy, 1978]. Individual bile acids in the methanolic layer were separated by TLC using chloroform: methanol: acetic acid: water (65:24:15:9, v/v) and visualized by spraying 1% phosphomolybdic acid in ethanol. Individual bile acids were quantified by densitometry using Camag TLC scanner (Model No III, Muttenz, Switzerland) as described [Sambaiah et al, 1986].

Uronic acid estimation

Uronic acid in methanolic layer of bile extract was estimated as described by Nelly and Gustav [1973]. An aliquot of methanolic layer of bile was made up to 0.5 mL with water followed by the addition of 3 mL of concentrated sulphuric acid and kept in a boiling water bath for 20 min and then cooled. Alcoholic carbazole (0.1%) 0.1 mL was added and kept in dark for 2 h and absorbance was measured at 530 nm against a blank sample. Uronic acid was calculated using glucuronic acid as reference standard.

Bile salt analysis in gallbladder bile

Mice bile was diluted with 0.1 N NaOH and extracted with chloroform: methanol according to Bligh and Dyer. The methanolic layer was filtered and then reduced to the volume of 0.5 mL under nitrogen and used for quantification of conjugated bile salts using methanol – 0.01 M KH_2P0_4 in the ratio 75:25 by HPLC analysis [Rosi et al, 1987].

Calculation of cholesterol saturation index (CSI)

Cholesterol saturation index for the bile was calculated by using the cholesterol, phospholipids and bile acids as input data using Windows 98, Microsoft Excel statistical programme. The coefficient for the fifth degree polynomial equation used in the program was taken from Carey's table [Carey, 1978].

Hydrophobicity index (HI)

Hydrophobicity index of bile was calculated based on the logarithms of bile salt capacity factors determined by using reverse phase high performance liquid chromatography. The standardized arbitrary indices of taurocholate and taurolithocholate were 0 and 1 respectively. The average hydrophobic-hydrophilic balance of biliary bile salts was quantified by means of bile salt monomeric hydrophobicity index [Heuman, 1989].

Fecal cholesterol analysis

Three days before the end of the experiment, feces were collected for fecal sterol analysis. The lipid was extracted from the feces using soxhlet extractor using petroleum ether for 6 h. The extract was filtered and concentrated to required volume and used for analysis.

Total cholesterol estimation

Cholesterol was estimated by the method of Searcy and Bergquist [1960]. An aliquot from the chloroform extract was dried under a stream of nitrogen followed by the addition of 1.5 mL of ferric chloride – acetic acid reagent. The reagent was prepared by diluting the stock reagent containing 504 mg/mL anhydrous $FeCl_3$ in 10 mL of glacial acetic acid to 1:100 dilution with glacial acetic acid. After mixing thoroughly, it was left at room temperature for 15 min. One mL of concentrated sulphuric acid was

added, mixed immediately on a vortex mixer and left at room temperature in the dark for 45 min. The color intensity of the clear solution was measured in a spectrophotometer (Shimadzu 160A model) at 540 nm. The cholesterol level in biological sample was estimated from the standard curve generated with reference cholesterol (30-150 μg).

HDL-cholesterol

HDL-cholesterol was determined afgter precipitation of apolipoprotein-B containing lipoproteins with heparin manganese chloride reagent [Warnick and Albers, 1978]. In brief, to 0.5 mL serum, 25 μL of heparin (5,000 units/mL) was added followed by the addition of 25 μL of 2 M manganese chloride (3.969 g/10mL). The solution was vortexed and kept at 4°C overnight. This was then centrifuged at 1100 X g for 20 min HDL cholesterol was measured as described earlier after extracting the supernatant with 3 mL of acetone: alcohol (1:1, v/v).

LDL+VLDL cholesterol estimation

The precipitate obtained from serum after adding heparin and manganese chloride contained LDL + VLDL. The precipitate was dissolved in 0.5 mL of saline and cholesterol was extracted in 3 mL acetone: alcohol (1:1, v/v) and cholesterol was estimated as described earlier.

Free cholesterol

Free cholesterol was estimated in the samples after precipitating it with digitonin [Sperry and Webb, 1950]. The precipitate was dissolved in solvent and cholesterol was determined as described earlier [Searcy and Bergquist, 1960]. The cholesterol esters content was obtained after subtracting free cholesterol from total cholesterol.

Phospholipids

Phospholipids were quantitated by ferrous ammonium thiocyanate method using di-palmitoylphosphatidyl choline as standard [Stewart, 1980]. The lipid extract in chloroform was evaporated under a stream of nitrogen and then dissolved in 2 mL of chloroform. Two mL of ferrous ammonium thiocyanate was added and vortexed for 1 min. After the phase separation, absorbance of chloroform phase was measured at 488 nm in Shimadzu 160 A spectrophotometer.

Triglycerides

Triglycerides were estimated [Fletcher, 1968] using tripalmitin as a standard. The lipid extract in chloroform was evaporated under a stream of nitrogen and then dissolved in 4 mL of isopropanol. Two g of triglyceride purifier was added, mixed well and centrifuged. Supernatant was transferred to another test tube, saponified with 0.6 mL of 5% potassium hydroxide in isopropanol: water (40:60, v/v) at 60°C for 15 min. After cooling, 1 mL of sodium metaperiodate prepared from the stock solution of 0.025 M in 1 N acetic acid (sodium metaperiodate (12 mL) and 20 mL of isopropanol and made up to 100 mL with 1 N acetic acid) was added, mixed and 0.5 mL of acetyl acetone was added, stoppered and incubated at 50°C for 30 min. After cooling to room temperature, the color intensity was read at 405 nm in Shimadzu 160 A spectrophotometer.

Serum lipid peroxides

Serum lipid peroxides were estimated by the method of Yagi [1984]. Serum (20 μL) was added to 4 mL of N/12 H_2SO_4 and shaken gently, followed by the addition of 0.5 mL of 10% phosphotungstic acid and mixed for 5 min at room temperature and centrifuged at 400 X g for 10 min. After discarding the supernatant, the sediment was mixed with 2 mL of N/12 H_2SO_4 and 0.3 mL of 10% phosphotungstic acid. The tubes were centrifuged at 400 X g for 10 min and sediment was suspended in 4 mL distilled water, 1 mL of TBA reagent (mixture of equal volumes of 0.6% TBA aqueous solution and glacial acetic acid) containing 20 nmoles butylated hydroxy anisole. The mixture was heated at 95°C for 60 min. Following this, TBA complex was extracted in 5 mL butanol and the fluoroscence measurement of butanol extract was carried out at an excitation wavelength of 515 nm and an emission wavelength of 553 nm using spectrofluorimeter (Hitachi F-2000, Japan).

Follwing, the above procedure, standards were prepared using 0.5 nmoles of 1,1,3,3-tetramethoxy propane with TBA reagent. Lipid peroxides were calculated using the formula –

Serum lipid peroxides = 0.5 X f/F X 1.0/0.02 nmoles/mL serum.

Where, F= Fluoroscence of tetramethoxy propane with TBA and

f = fluoroscence of sample

Lipid peroxides in liver

One g of liver was homogenized in 10 mL of 0.15 M KCl, in a Teflon homogenizer. The homogenate was filtered through cheesecloth and used for assay. Liver homogenate (4 mg protein) in 0.15 M KCl, 0.025 M tris HCl buffer pH 7.5, 2 mM adenosine diphosphate, and 10 μM ferrous sulphate were incubated at 37°C for 5 min. The reaction was initiated by adding 0.1 mM ascorbic acid and incubated at 37°C for 30 min (Buege and Aust, 1978). The final volume of the reaction mixture was 1 mL. The reaction was terminated using 2 mL of thiobarbituric acid (0.375% TBA, 15% TCA in 0.2 N HCl) containing 10 μM butylated hydroxyl anisole. Samples were heated for 15 min in a boiling water bath. Malondialdehyde (MDA) formed was measured at 535 nm and quantitated using an extinction coefficient of 1.56 X 10^{-5} $M^{-1}cm^{-1}$. The lipid peroxides were expressed as nmoles of malondialdehyde (MDA) formed / mg protein. Basal level of MDA was measured in the homogenate in which cofactors were not added [Buege and Aust, 1978].

Vitamin E

The tissues and serum were extracted with acetone and the protein precipitate was separated by centrifugation and the acetone was evaporated to dryness. The samples were resuspened in a known volume of acetone and tocopherols were quantitated using HPLC by employging C-18 Shimadzu HPLC column using solvent system; methanol:water 70:30(v/v) and the tocopherols were detected flourimetrically according to Zaspel and Csallany [1983].

Antioxidant enzymes in serum and liver

Liver was homogenized in appropriate buffer (1 g/10 mL) using galss homogenizer. It was filtered through cheesecloth and centrifuged at 600 X g for 15 min. The homogenate was used for the assay of antioxidant enzymes. Superoxide dismutase activity was measured by the inhibition of cytochrome C reduction mediated via superoxide anions generated by xanthine-xanthine oxidase and monitored at 550 nm [Flohe and Otting, 1984]. One unit of superoxide dismutase was defined as the amount required to inhibit the reduction of cytochrome-C by 50%. Catalase activity was assayed by following the decomposition of hydrogen peroxide at 240 nm [Aebi, 1984]. Glutathione peroxidase activity was determined by NADPH oxidation in a coupled reaction system consisting oxidized glutathione [Tappel, 1978].

HMG-CoA reductase activity

Preparation of liver microsomes

Animals were sacrificed between 9-10 p.m. by stunning. Liver was quickly excised, washed with isotonic saline and homogenized in 0.1M triethanolamine buffer pH 7.4 containing 0.02 M EDTA and 2 mM dithiothreitol and centrifuged at 1000 X g at 0-4°C for 15 min in a Sorvall RC 2B centrifuge. The supernatant was recentrifuged at 1,00,000 X g for 3 h in a Beckman LS-50B ultracentrifuge using type 65 rotor. The liver microsomal pellet was washed with 1 mL of triethanolamine hydrochloride buffer and homogenized with 2 mL of the same buffer and used immediately for enzyme activity measurement [Shapiro and Rodwell, 1971].

Enzyme assay

HMG-CoA reductase activity was measured by following the formation of monothiols with 5,5' dithiobis (2-nitrobenzoic acid) [Hulcher and Oleson, 1973]. To the 0.4 mL HMG CoA (6.486 mg/6 mL), 0.2 mL of dithiothreitol (DTT) (3.086 mg/2 mL) and NADPH 0.2 mL (50 mg/6mL) prepared in 0.1 M triethanolamine buffer containing 0.02 M EDTA and 2 mM DTT pH 7.4 was added to microsomes (~200 μg protein). The mixture was incubated at 37°C for 30 min and 20 μL of sodium arsenite (0.01 M) was added and after 1 min, the reaction was terminated by the addition of 0.01 mL of 2 M citrate buffer pH 3.5 containing 3% sodium tungstate to precipitate microsomal proteins. The mixture was again incubated at 37°C for 10 min. The solution was transferred to plastic tubes and centrifuged at 1000 X g for 15 min to remove proteins.

Five minutes before assaying the enzyme, 0.8 mL of the supernatant was mixed with 0.2 mL of tris buffer pH 10.6 and 0.1 mL of 2 M tris buffer pH 8.0 and 50 μL of 5 5'-dithiobis nitrobenzoic acid (DTNB) (3mM) was added, mixed thoroughly and absorbance was measured for 5 min at 412 nm (Shimadzu 160 A spectrophotometer). The absorbance due to monothiols was determined by extrapolating the linear portion of the curve after the addition of DTNB. The difference in absorbance between the complete reaction and that of all the components except NADPH represented the activity due to HMG CoA reductase.

Cholesterol 7α-hydroxylase and sterol 27-hydroxylase activities

Liver was excised, washed with isotonic saline and homogenized in buffer (0.20 M sucrose/10 mM HEPES pH 7.4). The homogenate was centrifuged for 10 min at 700

X g for 10 min. The supernatant was decanted and spun at 600 X g for 10 min and the resulting pellet was washed twice, and resuspended in homogenizing buffer. Sterol 27-hydroxylase and cholesterol 7α-hydroxylase were assayed by HPLC [Petrack and Latario, 1993].

Fatty acid analysis

Fatty acids were analysed as methyl esters prepared by using BF_3 – methanol [Morrison and Smith, 1964]. The fatty acid methyl esters were analyzed by gas chromatography (Shimadzu 14B fitted with FID) using fused silica capillary column 25 m x 0.25 mm (Parma bond FFAP-DF-0.25, Machery-Nagel Gm BH Co. Duren, Germany). The GC operating conditions were: injector temperature 240°C, detector temperature 260°C and column temperature was programmed at 180 – 240°C at 6°C rise/min. Individual fatty acids were identified by comparing with retention times of standards (Nu-Check prep. Inc., Elysian Minnesota, USA) and were quantitated by online chromatopack CR-6A integrator.

Activities of Alanine amino transferase (ALAT), Aspartate amino transferase (ASAT) and lactate dehydrogenase (LDH) in serum and liver

ALAT was assayed colorimetrically [Tonhazy et al, 1950] and ASAT activity was assayed according to Bergmeyer and Bernt [1974]. LDH activity was measured by following the rate of oxidation of NADH [Korenberg, 1974].

Protein estimation

Total proteins were measured according Lowry et al [1951] with BSA as a reference standard in all the experiments.

Determination of glycoprotein

Glycoprotein estimation was done according to Mantle and Allen [1978] using mucin as a reference standard. Samples were incubated for 2 h at 37°C with 0.2 mL freshly prepared periodic acid solution prepared by adding 10 μL of periodic acid to 10 mL of 7% acetic acid. After periodate oxidation, 0.2 mL of Schiff's reagent was added to the sample. It was allowed to stand for 30 min at room temperature and the color was read at 555 nm.

Microscopy

An aliquot of isotropic bile was observed under polarized microscope for precipitation and appearance of cholesterol monohydrate crystals.

Separation of biliary proteins by gel filtration

The concentrated rat bile was chromatographed on Sephadex G-100 column (75X1 cm). The elution buffer contained 10 mM Tris, 150 mM NaCl [Busch et al, 1991].

Separation of LMW glycoprotein by lectin affinity chromatography

The lyophilized LMW fraction obtained by gel filtration was solubilized in the eluting buffer and chromatographed on affinity columns [Busch et al, 1991].

Electrophoresis

Mini slab gel electrophoresis was performed according to Lamelli's procedure to determine the purity and approximate molecular weight of the isolated biliary proteins [Lamelli, 1970]. Running gels were 10% acrylamide in 0.375 M tris/HCl, 0.1% SDS at pH 8.8. Stacking gels were 4% acrylamide in 0.125 M Tris/HCl, 0.1% SDS at pH 7.8. Samples were dissolved with an equal volume of the sample buffer (0.1 M Tris/HCl pH 6.8), 2% SDS, 8% 2-mercapto-ethanol, 12% and 0.005% bromophenol blue) and then boiled for 10 min before loading. Low molecular weight protein kit was used as standard. Electrophoresis of the proteins was performed at a constant current of 40 mA over 5 h. Staining was done using comassie blue and destaining in acetic acid: methanol: water (10:25:65, v/v) overnight.

Preparation of model bile

Model biles of predetermined CSI were prepared according to the procedure of Kibe et al, [1985]. Sodium taurocholate in methanol was mixed with phosphatidyl choline and cholesterol in chloroform. The mixture was dried at 45°C under a mild stream of nitrogen and subsequently during 24 h under reduced pressure. The lipid film was reconstituted in 10 mM Tris/150 mM NaCl/3 mM NaN_3, incubated for 16h at 56°C and subsequently stored at 37°C in the dark under nitrogen.

Preparation of cholesterol monohydrate crystals (seed crystals)

Cholesterol monohydrate crystals were prepared according to Igimi and Carey [1981]. Five g of cholesterol was dissolved in 400 mL hot ethanol (95% v/v) and the solution was slowly cooled to room temperature and kept at 4°C for 3 days. Large cholesterol crystals were harvested by filtration (0.45 μm), washed with 100 mL water and resuspended in 50 mL water containing 3 mM NaN_3. The suspension was sonicated for 60 sec. The suspension was then centrifuged at 1000 X g for 5 min. The supernatant was collected and the pellet was resuspended and again sonicated

and centrifuged. The procedure was repeated until about 200 mL of supernatant was obtained. For preparation of seed crystals, the pooled cloudy supernatants were stored at 4°C for 4 days. During this period, a small amount of sediment formed. The supernatant was decanted and filtered through 0.8 μm filter. The filtrate was then passed through a 0.22 μm filter and the concentrate was then resuspended in water/NaN_3 (3 mM) thus producing a seed crystal suspension with crystal size ranging between 0.22 - 0.8 μm. The cholesterol concentration in the final seed crystal suspension was determined [Busch et al, 1991]

Separation of vesicles and micelles from model bile

The supersaturated model bile (CSI =1.5) was incubated with different proteins separated from rat bile at 37°C for 48 h. At the end of the incubation the model bile was subjected to gel permeation chromatography using sephadex-G 100 column (75X1 cm) and eluted with 10mM Tris buffer containing 150mM NaCl as solvent at a flow rate 1.0 mL/min. The fractions were collected separately and analyzed for proteins and lipids using standard procedures as described [Pattison et al, 1991].

Crystal growth assay

Aliquots of solutions of proteins of interest were added to screw capped vials. Filtered model bile was added to the vials. Both model bile and the vials with proteins were pre-equilibrated at 37°C before the two were mixed. The crystal growth assay was carried out in two sets viz: seeded and unseeded.

The crystal growth of seeded set was initiated by addition of 10 μg crystalline cholesterol whereas unseeded received no cholesterol. The aliquot of model bile mixture was taken out at different time intervals and diluted with tris-buffer saline (TBS). The absorbance at 900 nm was measured [Busch et al, 1991].

Calculation of crystal growth parameters

The crystal growth parameters were calculated from the crystal growth curves [Busch et al, 1991]. 1) Maximum growth rate [crystal growth index (I_g)] = maximal slope of experimental curve/maximal slope of control. 2) Final crystal concentration [crystal index (I_c)] = Final crystal concentration of experimental/final crystal crystal concentration of control and 3) Onset time of crystal detection [time index (I_t)] = onset time of experimental/onset time of control.

Statistical analysis

Statistical analysis was carried out using Windows 98 Microsoft excel statistical software and Prism Graphpad statistical software. Results were analyzed and the significance level was calculated using Tukey Kramer multiple comparison test and results are considered significant at $P<0.05$ and $p<0.01$.

Chapter – III

Studies on the hypocholesterolemic and antioxidant effects of sesame oil

Introduction

Sesame (S*esamum indicum L)* is one of the world's most important oil seed crops. It is not only a source of edible oil but seed itself provides a nutritious food for humans. It contains about 50% oil and 20 - 25% protein. Sesame oil (SO) is markedly different from other vegetable oils in many chemical, biological and physiological properties. SO is characterized by a very high oxidative stability compared with other vegetable oils. This unusual stability of SO is due to the endogenous antioxidants viz; sesamol, sesamin, and sesamolin (lignans) and tocopherols. Sesaminol is produced from sesamolin, a minor constituent of sesame oil by intermolecular transformation during industrial bleaching process of unroasted sesame oil. Sesamol is liberated from sesamolin during the frying process. In contrast, sesamin another minor component of SO, gives episesamin during the bleaching process. Sesame oil and its natural antioxidants are gaining wide attention for the role they may play on lipid metabolism especially in cholesterol metabolism [Sugano et al, 1990; Hirose et al, 1991; Umeda-Sawada et al, 1994].

Coronary heart diseases (CHD) have been increasing alarmingly in recent times in India and in other developing countries. Efforts towards finding a safe and effective hypocholesterolemic agent have been continuing. If a dietary constituent can act as a hypocholesterolemic agent, it is all the more advantageous. Sesame oil is likely to fit into this category very well. It is an excellent source of good edible oil and can also provide natural antioxidant cover. The physiological importance of sesame oil with its antioxidant lignans has not been clearly established especially as a hypocholesterolemic agent.

Cholesterol has been implicated as a causative factor in atherosclerosis. The total body cholesterol homeostasis is controlled by supply and removal pathways. Liver is an important organ in the metabolism of cholesterol and is also sensitive to changes in the dietary lipids. β-Hydroxy β-methylglutaryl CoA (HMG CoA) reductase (EC No. 1.1.1.34) (Mevalonate: NADP oxidoreductase) is one of the important enzymes in the biosyntheis of cholesterol. It converts HMG CoA to mevalonate, the committed step

in the biosynthesis of cholesterol. It is one of the most highly regulated enzymes in nature and is controlled through a multivalent feed back mechanism.

Cholesterol is converted into bile acids in liver and excreted in bile. Many studies have shown that dietary fatty acids influence bile acid secretion. Bile acid synthesis is regulated by cholesterol 7α-hydroxylase enzyme in the liver and increase in the bile acid synthesis and its excretion in bile, preventing bile acid re-absorption in the gut are known to decrease serum cholesterol levels [Gibbons et al, 1982].

Lipid peroxidation may occur in any lipid environment containing PUFA exposed to oxidants capable of abstracting bis-allylic hydrogen atom. The subsequent reaction of the pentadienyl radical with oxygen to form the peroxyl radical results in a chain reaction which in turn greatly amplifies the severity of the initial oxidation. The production of oxygen derived free radicals is involved in the onset of many diseases such as cancer, rheumatoid arthritis and atherosclerosis as well as in degenerative processes associated with aging [Hertog et al, 1995; Halliwell, 1995; Steinberg, 1995; Dormady, 1983]. Reactive oxygen species (ROS) have been implicated in the mechanism of the damage to membranes due to the presence of many unsaturated lipids. ROS are generated in biological systems through metabolic processes and exogenous sources such as food components, drugs, ultraviolet light, ionizing radiation and pollution [Sanueni et al, 1983]. According to generally accepted mechanisms, major deleterious effects are caused by the hydroxyl radical (OH^-) generated from hydrogen peroxide and by the superoxide species in the presence of redox active transition metals [Cherion, 1988; Briviba and Siesm, 1994]. Many defense mechanisms have developed in living organisms to limit the levels of ROS and the damage they inflict. Included among them are endogenous enzymes such as superoxide dismutase, catalase, and glutathione peroxidase [Ames et al, 1993].

Antioxidants play a vital role in protecting the body from oxidative damage. Numerous studies have been shown that cellular free radicals damage can be repaired by natural antioxidants. Antioxidants may quench free radicals, change their redox state, be targeted for destruction, regulate oxidative processes involved in signal transduction, affect gene expression pathways of cell proliferation, differentiation and death. Some of the compounds present in plants such as ascorbic acid, tocopherol, thiols, bioflavanoids, carotenoids etc; are proved to be potent antioxidants. SO contains unique unsaponifiable constituents viz; sesamol, sesamin,

and sesamolin (lignans) and tocopherols, which play an important role in antioxidant defense mechanism [Fukuda et al, 1986; Yamashita et al, 1992].

Excessive iron has been shown to cause toxic effects resulting in health problems [Bristton et al, 1987] due to the lack of excretion of excessive iron by body. Iron overloading leads to chronic diseases, impaired cardiac function, diabetes mellitus, endocrinopathies, skin pigmentation etc [Niederen et al, 1985]. Hepatotoxicity is the most common finding in patients with iron overloading. This is due to the massive deposition of iron in hepatic parenchymal cells, which eventually produces fibrosis and ultimately results in cirrhosis [Weintraub et al, 1985]. In experimental animals iron overload conditions can be obtained by injection of iron salts intraperitoneally [Dillard et al, 1984]. One of the mechanisms by which iron induces the toxicity is by increasing oxidative stress and lipid peroxidation. The iron overload increases the formation of ethane, pentane, urinary malondialdehyde, tissue TBARS and conjugated dienes [Dillard et al, 1984]. Assessment of ALAT, ASAT and LDH in serum gives an indication of the extent of liver damage [Wills, 1985].

The present study will highlight the importance of sesame oil apart from as a dietary constituent (oil) but also as a source of cholesterol lowering agent and natural antioxidant. The influence of sesame oil on cholesterol levels in liver and serum, distribution among various lipoprotein fractions, its absorption and excretion will be of importance for understanding the health beneficial effects of sesame oil.

The present study also evaluates the protective role of sesame oil as an antioxidant in normal and iron induced lipid peroxidation and hepatotoxicity.

Experimental –

Studies on hypocholesterolemic effect of sesame oil

Sesame oil is traditionally known for its health beneficial effects. In the present study the hypocholesterolemic and antioxidant effects of sesame oil were studied. Thirty-six male Wistar rats (28 day old) were grouped into 3 groups with 12 animals/group. High cholesterol diet (HCD) was prepared by the addition of 0.5% cholesterol and 0.25% bile salts to the AIN-76 basal diet as described earlier. GNO (groundnut oil) and SFO (sunflower oil) were used in the study for comparative purposes.

Each group was sub-divided into 2 sub-groups with 6 animals each and one sub-group fed with normal diet and the other with HCD diet of different vegetable oils for 8 weeks. During the study, growth and food intake were monitored and FER was calculated as the ratio of weight gained by the animal to the food consumed. After the experimental period, the animals were sacrificed under ether anesthesia. Blood was collected and serum was separated as described previously. The serum and liver lipid parameters were estimated as described. Serum and liver lipid peroxides and vitamin-E were also estimated as described previously. The HMG-CoA reductase enzyme activity was measured in microsomes.

Studies on the influence of sesame oil on bile composition in rats

Cholesterol is excreted mainly through bile and to a little extent in the feces as such from the body. In order to understand the mechanism of hypocholesterolemic effect of sesame oil, absorption of cholesterol from the intestine and excretion of cholesterol in bile was studied. Cholesterol absorption was calculated by taking the amount of cholesterol intake and amount of cholesterol excreted in the feces.

In this experiment, 30 male Wistar rats weighing about 180 g were grouped into 10 animals/group and fed with SO, GNO and SFO diets for six weeks. At the end of experimental period the bile was collected after bile duct cannulation as described and the biliary parameters were determined.

Studies on the antioxidant effect of sesame oil in rats

Sesame oil is known to possess antioxidant property and this characteristic feature was attributed to the presence of sesame lignans and tocopherols present in the oil. In this experiment antioxidant effect of sesame oil was evaluated by inducing lipid peroxidation in rats by injecting iron intraperitonially.

Twenty-four male Wistar rats (28 day old) were grouped into 12 animals/group and fed with SO and GNO diet for 8 weeks. At the end of experimental period, 6 rats from each group were intraperitonially injected with $FeSO_4$ (30 mg/kg b.w) in saline and the other 6 rats were injected with 1 mL saline alone 1h before dissection.

The serum, liver homogenate and liver microsomal lipid peroxides were estimated as described. The liver and serum antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase were also determined. The ALAT, ASAT and LDH activities were also determined.

Results

Studies on hypocholesterolemic effect of sesame oil

Physiological effects of sesame oil on growth, food intake and lipid parameters were studied. Groundnut oil fed group served as a control group and for comparative purposes sunflower which is rich in linoleic acid was used. Similar studies were conducted in rats fed lithogenic diet containing cholesterol.

Fatty acid composition and other non-saponifiables of sesame oil

The vegetable oils are chiefly comprised of triglycerides. The fatty acid composition of the triglyceride and the position of the fatty acids in the glycerol backbone plays an important role in the nutritional quality of oil. Hence, the fatty acid composition of sesame oil (SO), groundnut oil (GNO) and sunflower oil (SFO) were determined and the resultes are given in table 3.1. The oleic acid content was 37.26, 42.57 and 30.12% in SO, GNO and SFO respectively. Similarly the linoleic acid content was 40.25, 34.40 and 57.20% in SO, GNO and SFO respectively. The sesamol content was 82.46 mg/100 g and sesamin was found to be 27.23 mg/100 g sesame oil. These lignans are present only in SO.

Table 3.1: Fatty acid composition of diets containing sesame oil, groundnut oil and sunflower oil

Fatty acid	SO	GNO	SFO
(%) fatty acids			
16:0	13.37±1.26	12.26±1.12	6.30±1.12
18:0	2.50±0.03	3.00±0.11	4.00±0.38
18:1	37.26±4.28	42.57±5.13	30.12±5.21
18:2	40.25±3.93	34.40±3.29	57.20±3.89
20:0	1.87±0.06	3.6±0.21	1.20±0.11
22:0	3.00±0.12	1.5±0.03	ND
Unsaponifiables in sesame oil			
Sesamol (mg/100g)	82.46±6.83		
Sesamin (mg/100g)	27.23±3.28		

Values are mean ± SD of 3 samples carried out in duplicate

SO – sesame oil, GNO – groundnut oil, SFO – sunflower oil

ND – Not detected

Effect of sesame oil on growth parameters

Sesame oil was incorporated in the diet at 10.0%. The fat level was kept constant in all the groups. Groundnut oil was chosen for control diets since it is most commonly consumed edible oil in Southern India. Table 3.2 shows the growth parameters like food intake, body weight gain and FER (food efficiency ratio) and liver weight. Food intake and gain in body weight were comparable in all groups except in rats fed SFO diet with or without cholesterol where food intake was significantly higher. SO significantly increased relative liver weight compared to other oils with or without cholesterol in the diet.

Effect of sesame oil on serum and liver lipids

The serum lipids reflect various pathological and diseased conditions. Similarly, liver is the major site for lipid metabolism and is also sensitive to changes in the dietary lipids. Hence serum and liver lipid profiles can be taken as markers for ascertaining disease and metabolic alterations in the body.

The serum lipid profile is given in Table - 3.3. SO reduced the concentration of serum total cholesterol both in normal and cholesterol fed rats by 8, 11, 15 and 26% respectively when compared to GNO and SFO groups. HDL – cholesterol content was increased in SO fed rats but it was not statistically significant. On the other hand SO significantly reduced the LDL-cholesterol by 25% in normal and 21% in cholesterol fed rats compared to GNO fed animals. Serum triglycerides were significantly lowered by SO compared to GNO fed animals in both control and cholesterol fed state. Serum phospholipid content was increased by 11 and 6% in normal and cholesterol fed rats by SO compared to GNO fed rats.

The liver cholesterol was significantly lowered in SO fed rats by 18, 37, 11 and 28 % in normal and cholesterol fed rats compared to GNO and SFO fed rats respectively (Table-3.4). The reduction was apparently visible in both free as well as esterified cholesterol fractions. The liver triglyceride content was significantly lowered by SO in cholesterol fed group compared to SFO fed group. Phospholipid content was marginally increased but it was not statistically significant.

Table 3.2: Effect of feeding sesame oil on food intake, gain in body weight, food efficiency ratio (FER) and liver weight in normal and cholesterol fed rats

Groups	SO		GNO		SFO	
	Normal	HCD	Normal	HDC	Normal	HCD
Food intake (g)	644.2 ± 10.1	630.2 ± 9.4	665.0 ± 13.6	634.7 ± 8.6	698.6 ± 9.4[a]	690.5 ± 11.3[a]
Gain in b. wt. (g)	211.8 ± 13.4	227.6 ± 7.2	214.2 ± 9.2	226.8± 10.3	198.0± 14.7	235.3 ± 20.1
FER	0.32 ± 0.02	0.36 ± 0.03	0.32 ± 0.01	0.35 ± 0.02	0.28 ± 0.03	0.34 ± 0.01
Liver wt. (g/100g b.wt)	3.60 ± 0.31	5.21 ± 0.31	2.94 ± 0.42	4.68 ± 0.22[b]	3.10 ± 0.13[b]	4.66 ± 0.41[a]

Values are mean ± SD of 6 rats/group.

Statistically significant when compared to respective SO group at

[a]P<0.01, [b]P<0.05

FER- Food Efficiency Ratio. (FER was calculated as the ratio of weight gained by the animal to the food consumed).

Table 3.3: Effect of feeding sesame oil on serum lipids in normal and cholesterol fed rats

Groups	SO		GNO		SFO	
	Normal	HCD	Normal	HCD	Normal	HCD
	mg/dL					
TC	68.4 ± 6.3	183.2 ± 6.2	73.7 ± 3.9	211.4 ± 4.7[a]	76.1 ± 4.1	230.3 ± 5.6[a]
HDL-C	40.6 ± 3.9	18.2 ± 3.2	36.7 ± 5.6	22.3 ± 2.1	39.2 ± 6.3	21.1 ± 3.2
VLDL + LDL-C	27.8 ± 3.7	164.9 ± 6.2	37.0 ± 3.3[b]	189.1 ± 3.9[a]	36.9 ± 4.1[b]	209.1 ± 4.3[a]
CE	42.0 ± 3.2	142.9 ± 3.2	42.5 ± 2.9	175.2 ± 4.1[a]	43.9 ± 4.1	193.0 ± 3.8[a]
FC	26.3 ± 4.3	40.3 ± 2.3	31.2 ± 4.3	36.2 ± 3.1	32.2 ± 2.6	37.3 ± 2.3
PL	136.2± 3.9	140.3± 4.3	120.2 ± 2.2[a]	132.3 ± 2.3[b]	121.6 ± 2.1[a]	134.3 ± 4.1
TG	102.6 ± 4.4	116.2 ± 4.3	112.7 ± 5.3[b]	128.3 ± 3.9[a]	120.6 ± 3.9[a]	130.6 ± 4.1[a]

Values are mean ± SD of 6 rats/group.

Statistically significant when compared to respective SO group at [a]P<0.01, [b]P<0.05.

TC-total cholesterol, HDL-cholesterol, VLDL+LDL-cholesterol, CE-cholesterol esters, FC-free cholesterol, PL-phospholipids, TG-triglycerides.

Table 3.4: Effect of feeding sesame oil on liver lipids in normal and cholesterol fed rats

Groups	SO		GNO		SFO	
	Normal	HCD	Normal	HCD	Normal	HCD
	mg/g					
TC	3.26 ± 0.2	18.56 ± 2.3	4.01 ± 0.3[a]	20.79 ± 3.1	5.18 ± 0.4[a]	25.66 ± 2.9[a]
CE	1.14 ± 0.1	12.24 ± 1.3	1.82 ± 0.1[a]	15.37 ± 1.6[b]	2.92 ± 0.1[a]	20.63 ± 1.8[a]
FC	2.12 ± 0.2	6.32± 1.2	2.18 ± 0.1	5.42 ± 0.9	2.26 ± 0.2	5.03 ± 0.6
PL	10.31 ± 1.22	11.50 ± 1.61	9.49 ± 1.62	9.72 ± 1.32	8.77 ± 1 .31	10.82 ± 2.13
TG	4.08 ± 0.72	14.69 ± 1.61	4.20 ± 0.62	17.17 ± 2.13	4.26 ± 0.39	19.09 ± 1.76[b]

Values are mean ± SD of 6 rats/group.

Statistically significant when compared to respective SO group

at [a]P<0.01, [b]P<0.05

TC-Total cholesterol, CE-cholesterol esters, FC-Free cholesterol, PL-phospholipids, TG-triglycerides

Effect of sesame oil on HMG-CoA reductase activity

The HMG-CoA reductase is the key regulatory enzyme in cholesterol biosynthesis. The activity of this enzyme was estimated in liver microsomes (Fig-3.1). It showed that SO feeding significantly lowered the HMG-CoA reductase activity by 65 and 69% in normal and cholesterol fed state respectively compared to GNO and SFO fed animals.

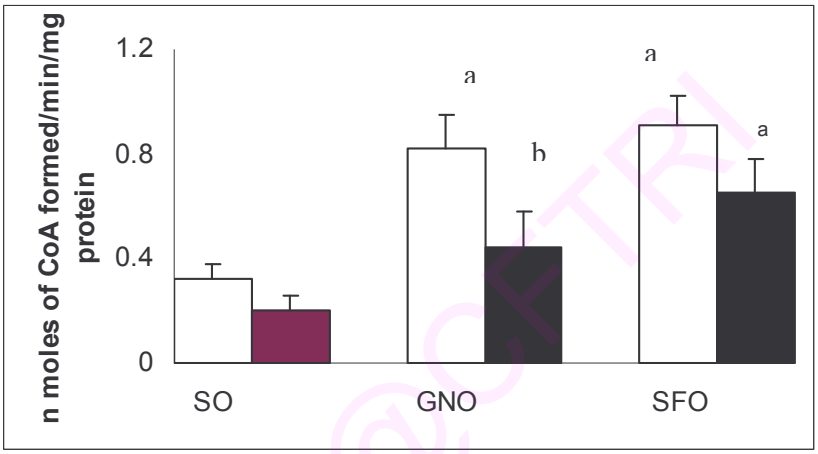


Fig 3.1: Effect of sesame oil on HMG-CoA reductase activity in normal and cholesterol fed rats

Values are mean ± SD of 6 rats / group

□ Normal group ■ Cholesterol fed group

Statistically significant when compared to respective SO group at $^{a}P<0.01$, $^{b}P< 0.05$

Effect of sesame oil on cholesterol absorption

The cholesterol homeostasis in the body is controlled by supply and removal pathways. Hence, the effect of SO on the absorption of cholesterol in rats was studied by balance method over a period of three days by following the intake and excretion of dietary cholesterol. The results presented in the Table - 3.5 shows that % absorption of added cholesterol in the diet was lowest in SO group. On the other hand the amount of cholesterol excreted was significantly higher in SO fed group.

Table 3.5: Effect of feeding sesame oil on cholesterol absorption and excretion in normal and cholesterol fed rats

Groups	HCD			Normal	
	Intake	Excretion	Absorption (%)	Intake	Excretion
	(mg / rat/ day)				
SO	67.58 ± 4.20	27.87 ± 2.16	58.72 ± 1.37	-	4.33 ± 1.19
GNO	68.03 ± 3.20	14.69 ± 3.12[a]	78.40 ± 2.17[a]	-	3.24 ±1.23[b]
SFO	68.75 ± 6.12	7.96 ± 2.42[a]	88.43 ± 1.17[a]	-	2.72 ± 0.91[a]

Values are mean ± SD of 6 rats/group.

Statistically significant when compared to respective SO group

at [a]P<0.01, [b]P<0.05

Studies on the influence of sesame oil on bile composition in rats

Effect of sesame oil on bile composition

Bile plays an important role in the digestion and absorption of lipids. It is one of the important routes through which cholesterol and its metabolites (bile acids) are excreted. Hence, the effect of SO on bile flow and its constituents was evaluated (Table-3.6). SO marginally enhanced bile flow and bile solids, but both were not statistically significant. The cholesterol content in bile was significantly higher by 57% in SO fed animals compared to GNO. Total and individual bile acids were not influenced by SO. On the other hand, phospholipid content was increased by 20% whereas uronic acid and protein content in bile were increased marginally in animals fed SO, but it was not statistically significant.

The biliary phospholipids fatty acid composition was given in (Table-3.7). The results showed that the SO increased the biliary linoleic acid content in the biliary phospholipids.

Table 3.6: Influence of sesame oil on bile secretion and composition

Parameters analysed	SO	GNO	SFO
Bile flow (ml/h)	0.68 ± 0.04	0.57 ± 0.06	0.63 ± 0.03
Bile solids (%)	3.18 ± 0.18	3.15 ± 0.21	2.63 ± 0.41
Cholesterol (μ moles/h)	0.66 ± 0.02	0.42 ± 0.06 [a]	0.51 ± 0.05 [a]
Phospholipids (μ moles/h)	1.09 ± 0.05	0.88 ± 0.09	0.87 ± 0.02 [a]
Uronic acid (μ moles/h)	2.13 ± 0.19	1.53 ± 0.09 [a]	1.60 ± 0.08 [a]
Protein (mg/h)	5.13 ± 0.42	3.46 ± 0.42	3.92 ± 0.27
Total bile acids (μ moles/h)	11.05±1.08	11.49±1.64	10.44 ±0.72
Taurocholic acid (μ moles/h)	4.82 ± 0.76	4.70 ± 0.57	4.46 ± 1.03
Taurodeoxycholic acid (μ moles/h)	2.84 ± 0.39	3.10± 1.21	2.93 ± 0.79

Values are mean ± SD of 6 rats /group.

[a] Statistically significant when compared to SO group at P <0.05

Table 3.7: Effect of sesame oil on biliary phospholipid fatty acid composition

Fatty acid	SO	GNO	SFO
16:0	35.00±3.60	37.83±2.87	38.69±2.60
18:1	14.27±2.86	15.34±2.86	15.17±2.17
18:2	35.14±2.38	30.84±4.00[a]	30.51±3.06 [a]
20:4	15.59±1.83	15.80±1.52	15.06±1.98 [a]

Values are mean ± SD of 6 rats /group.

[a] Statistically significant when compared to SO group at P<0.05

Effect of sesame oil on serum and liver lipid peroxides

The free radicals generated during various metabolic processes causes lipid peroxidation, which is deleterious to health. Serum and liver lipid peroxide levels gives an idea about the lipid peroxide levels in the body. Serum lipid peroxides were significantly lower in SO group by 52 and 34 % in normal animals compared to GNO and SFO fed animals respectively (Fig-3.2). Similarly liver lipid peroxides were significantly lowered in SO group compared to GNO and SFO group (Fig-3.3). Feeding of cholesterol increased the basal levels of peroxides in both serum and liver. SO feeding resulted in lower levels of peroxides in both serum and liver was statistically significant only in SFO group.

Effect of sesame oil on ∝-tocopherol content

Sesame oil is a rich source of tocopherols. The tocopherols are known to play a vital role as an antioxidant and hence the α-tocopherol content was estimated. The α-tocopherol content in the serum was higher in animals fed SO by 16% each in normal and cholesterol fed animals respectively compared to GNO fed animals. The liver α-tocopherol content was also higher by 18 and 9% in normal and cholesterol fed animals respectively compared to GNO fed animals. Whereas, no significant change in the levels of γ-tocopherel in serum and liver was observed (Table-3.8).

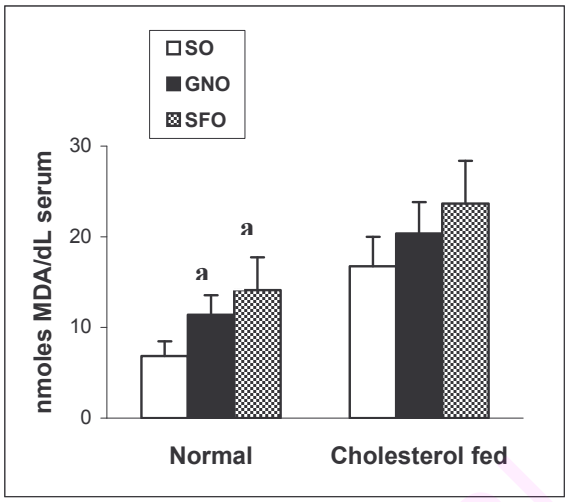


Fig 3.2: Effect of sesame oil on serum lipid peroxides in normal and cholesterol fed rats

Values are mean ± SD of 6 rats/group

[a] Statistically significant different when compared to respective SO group at P< 0.01

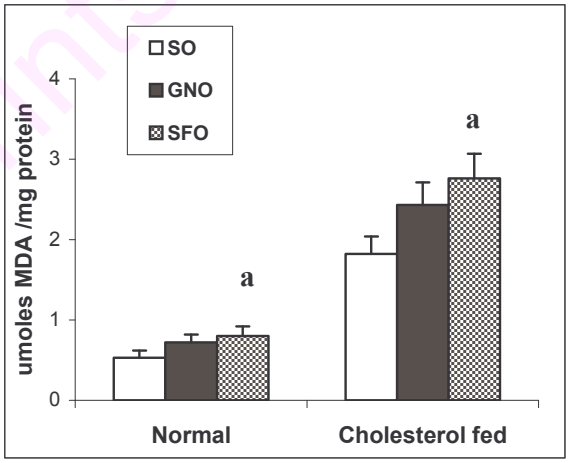


Fig 3.3: Effect of sesame oil on liver lipid peroxides in normal and cholesterol fed rats

Values are mean ± SD of 6 rats/group.

[a] Statistically significant different when compared to SO group at P<0.01

Table 3.8: Effect of feeding sesame oil on tocopherol level in normal and cholesterol fed rats

Groups	SO		GNO		SFO	
	Normal	HCD	Normal	HCD	Normal	HCD
α-tocopherol (μmoles /dL serum)	0.73 ± 0.12	1.30 ± 0.13	0.63 ± 0.16	1.12 ± 0.32	0.60 ± 0.11[b]	1.08 ± 0.26
γ-tocopherol (μmoles /dL serum)	0.18 ± 0.02	0.32 ± 0.12	0.15 ± 0.03	0.26 ± 0.09	0.15 ± 0.02	0.26 ± 0.10
α-tocopherol (μmoles/g liver)	35.4 ± 2.3	44.5 ± 1.75	30.03 ± 2.60[b]	40.9 ± 1.36[b]	29.75 ± 1.80[a]	38.8 ± 2.16[a]
γ-tocopherol (nmoles/g liver)	1.71 ± 0.23	2.28 ± 0.32	1.59 ± 0.19	2.17 ± 0.24	1.43 ± 0.32	1.98 ± 0.20

Values are mean ± SD of 6 rats/group.

Statistically significant when compared to respective SO group at [a]P<0.01, [b]P<0.05.

Studies on the influence of sesame oil on iron induced lipid peroxidation in rats

Effect of sesame oil on iron induced lipid peroxidation in rats

Fe^{2+} injection significantly increased lipid peroxidation in serum, liver homogenate and microsomal suspension. Rats injected with saline alone did not show significant difference in lipid peroxidation indicating the importance of Fe^{2+} in the induction of lipid peroxidation. Serum lipid peroxides were significantly lower (20%) in sesame oil fed animals compared to GNO fed animals (Fig 3.4). Basal TBARS values in liver homogenate were not altered with iron injection but incubation with cofactors significantly (about 3 times) increased. In animals fed with sesame oil the increase in TBARS in liver homogenate was significantly lower compared to GNO fed animals (Fig 3.5). Liver microsomal basal TBARS in levels were three fold higher than in liver homogenate (Fig 3.6). On incubation with cofactors, the TBARS levels were increased significantly in GNO fed animals. Sesame oil feeding significantly lowered the TBARS levels both in basal as well as in induced conditions (Fig 3.6).

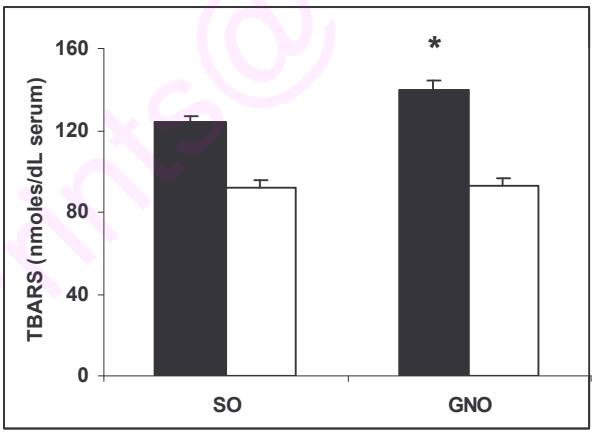


Fig 3.4: Effect of sesame oil on iron induced lipid peroxidation in rat serum

Rats were injected with 1mL saline (□) or injected with $FeSO_4$, 30mg/kg body weight in 1 mL saline (■)

Values are mean ±SD of six rats/group

*Statistically significant when compared to respective SO group at $p<0.01$

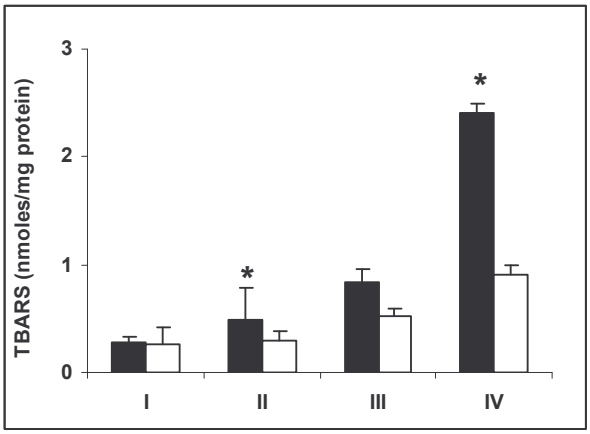


Fig 3.5: Effect of sesame oil on lipid peroxidation in rat liver homogenate

Rats were injected with 1 mL saline (□) or $FeSO_4$, (30mg/kg body weight) in 1 mL saline. (■) (bars I and III SO fed groups and II & IV GNO fed groups). Lipid peroxidation was measured at zero mins (bars I and II) and at 60 min (bars III and IV) of incubation as described in materials and methods. Values are mean ± SD for six rats/group. *Statistically significant when compared to respective SO group at p<0.01

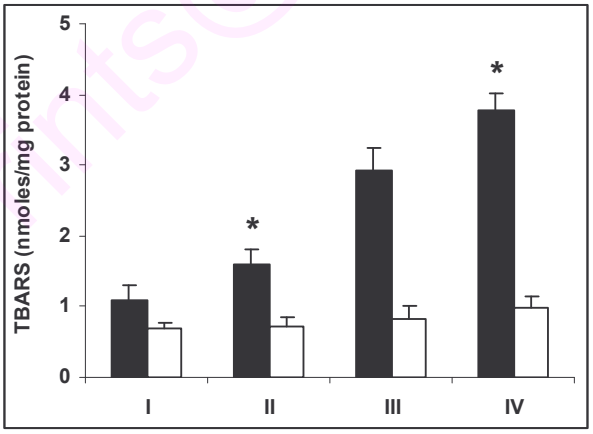


Fig 3.6: Effect of sesame oil on lipid peroxidation in rat liver microsomal suspension

Experimental conditions are similar as described in fig 3.5.

Values are mean ± SD of six rats/group.

*Statistically significant when compared to respective SO group at p<0.01

Effect of sesame oil on antioxidant enzymes in iron induced lipid peroxidation in rats

The effect of sesame oil on the activities of the antioxidant enzymes: superoxide dismutase, catalase and glutathione peroxidase were measured in liver and serum. The activities of these enzymes in liver were higher in rats fed with sesame oil diet (Table 3.9). Injection of iron enhanced the antioxidant enzyme levels. SO feeding further increased the levels of these antioxidant enzymes. Similar results were observed in serum (Table 3.10). These studies indicated that sesame oil in the diet lowers the lipid peroxidation by maintaining high-level activities of antioxidant enzymes and also by protecting the liver damage caused by iron to some extent.

Table 3.9: Effect of sesame oil on antioxidant enzymes in rat liver homogenate

Enzyme	GNO+Fe^{2+}	GNO	SO+Fe^{2+}	SO
SOD ♦	124.0±1.6*	106.4±2.1	139.4±2.6	110.0±3.4
Catalase#	204.8±1.2*	193.4±3.0	258.7±1.32	200.3±4.20
Glutathione peroxidase •	3.61±0.26*	3.47±0.17*	5.65±0.31	4.64±0.41

Values are mean ± SD for group of 6 rats

*Statistically significant when compared to respective SO group at $p<0.01$

Experimental conditions are same as described in Fig.3.5

♦ (Units/mg protein) 1 unit of SOD was defined as the amount required to inhibit the reduction of Cyt-C by 50%

(μ moles H_2O_2 decomposed/mg protein)

• (m units/mg protein) 1m unit = 1μ mole NADPH oxidized

Table 3.10: Effect of sesame oil on antioxidant enzymes in rat serum

Enzyme	GNO+Fe^{2+}	GNO	SO+Fe^{2+}	SO
SOD♦	1.33±0.29	1.27±0.13*	1.34±0.21	1.14±0.12
Catalase#	150.0±1.8*	110.1±1.1*	160.2±1.2	130.1±1.37
Glutathione peroxidase•	16.57±1.12*	9.78±1.02*	22.01±1.03	16.58±1.36

Values are mean ± SD for group of 6 rats

`*Statistically significant when compared to respective SO group at p<0.01

Experimental conditions are same as described in fig. 3.5

♦ (Units/mg protein) 1 unit of SOD was defined as the amount required to inhibit the reduction of Cyt-C by 50%

(nmoles H_2O_2 decomposed/mg protein)

• (m units/mg protein)1m unit = 1 nmole NADPH oxidized

Effect of sesame oil on activity of ALAT, ASAT and LDH in iron induced lipid peroxidation in rats

Activities of ALAT, ASAT and LDH in serum will reflect the physiological status of liver function. Hence the activities of ALAT, ASAT and LDH were assayed and results were presented in table-3.11. Results indicated that serum ALAT, ASAT and LDH levels were lower in sesame oil fed animals than in groundnut oil fed diet group. The ALAT, ASAT and LDH concentration were 26%, 21% and 75% higher respectively in GNO fed group injected with iron compared to SO fed group indicating lesser damage to liver upon feeding sesame oil after iron injection.

Table 3.11: Effect of sesame oil on serum enzymes in rats injected with saline or iron

Enzyme	GNO+Fe^{2+}	GNO	SO+Fe^{2+}	SO
ALAT[1]	373.9±5.11*	148.3±9.55*	252.6±1.39	111.4±9.15
ASAT[1]	186.9±3.92*	87.8±2.31*	148.7±2.88	77.4±2.99
LDH[2]	270.2±6.34*	77.1±2.03*	186.4±6.77	67.6±2.65

Values are mean ± SD for group of 6 rats

*Statistically significant when compared to respective SO group at P<0.01

Experimental conditions are same as described in Fig.3.5

[1] μ moles of pyruvate formed /dL serum

[2] μ moles of NADH/min/dL serum

Discussion

Sesame seed is traditionally used as health food and has been considered to elicit medicinal value for the prevention of certain degenerative diseases such as atherosclerosis, aging, hypertension etc, in several Asian countries. Most of the studies have been focused on sesame lignans on their potential as an antioxidant [Fukuda et al, 1986; Yamashita et al, 1992], anticarcinogenicity [Hirose et al, 1992], anti-hypertensive effect [Matsumura et al, 1995; Kita et al, 1995] and in alleviation of hepatic injury caused by alcohol and carbon tetrachloride in mice [Akimoto et al, 1993]. Sesame lignans with their natural antioxidant activity are gaining wide attention for the role they may play on lipid metabolism especially cholesterol metabolism [Sugano et al, 1990; Hirose et al, 1991: Umeda-Sawada 1994]. Except for a stray report [Koh et al, 1987] that sesame oil tended to reduce the serum cholesterol level in rats compared to corn oil, there are no reports about nutritional benefits of sesame oil. Most of the beneficial effects on lipid metabolism have been carried out with isolated lignans especially with 0.5% sesamin in the diet. There were no studies to confirm whether these benefits can also be found in whole sesame oil. The present study was focused on the nutritional benefits and health implications of SO. Hypercholesterolemia is termed as a risk factor for atherosclerosis and the hypocholesterolemic and antioxidant effect of SO at 10% level with or without cholesterol in the diet was examined here for its effect on lipid metabolism and peroxidation.

The sesame oil used in the experiment contained 27.23 mg/100 g oil [Table-3.1] corresponding to 0.0027% sesamin in the diet. SO feeding to rats affected neither the gain in body weight nor food intake but increased relative liver weight [Table-3.2] with slight increase in liver phospholipids. Activities of ALAT, ASAT and LDH in serum [Table-3.11] were lower indicating that there was no pathological significance of this observation. Sesame oil reduced both the serum and liver cholesterol and triglyceride levels in normal and cholesterol fed rats. Cholesterol supplementation resulted in elevated LDL-cholesterol level and SO significantly prevented this rise in LDL – cholesterol. Cholesterol level in the body is regulated by the intake from the diet and its efficacy of absorption, its biosynthesis in the body, and catabolism of cholesterol in the body. Cholesterol fed rats in all the groups on an average consumed about 68.12 mg/day of cholesterol. But the absorption was significantly lowered in SO group [58.7%] compared to 78.4 and 88.4% in GNO and SFO fed groups respectively indicating significant inhibition of cholesterol absorption in SO fed

animals which is in agreement with earlier reports of inhibition of cholesterol absorption by sesamin at 0.5% [Hirose et al, 1991].

Liver is the primary site for the biosynthesis of cholesterol in which HMG-CoA reductase plays an important role as the rate limiting regulatory enzyme. It has been shown earlier that sesamin at 0.5% level in the diet inhibited the HMG-CoA reductase activity [Hirose et al, 1991]. In this study, SO inhibited HMG-CoA reductase activity in liver microsomes not only in rats fed cholesterol free diet but also in cholesterol fed state indicating cholesterol biosynthesis is also affected by SO. In the present study, eventhough the sesamin content in the SO group is 0.0027%, still SO inhibited the HMG-CoA reductase activity. Thus the hypocholesterolemic effect of SO can be explained by the inhibition of intestinal absorption of cholesterol and partly by inhibiting biosynthesis of cholesterol by reducing the activity of HMG-CoA reductase activity in liver.

Bile is an important route for the excretion of cholesterol and its metabolites. SO feeding increased the total bile flow and bile solids content in the bile. The cholesterol and bile acids excretion in the bile was increased by SO. The cholesterol 7α-hydroxylase enzyme which is involved in the synthesis of bile acids from cholesterol activity was increased by 0.5% sesamin. It also increased the fecal sterol excretion and inhibited absorption of cholesterol [Hirose et al, 1991]. It is assumed that the rate of synthesis of bile acids nearly always are paralleled by corresponding changes in the rate of cholesterol biosynthesis in liver [Danielsson and Sjovall, 1975].

Fatty acids utilized for esterification are derived from the serum free fatty acids synthesized *de-novo* and intrahepatic lipolytic process. In the present study the decreased levels of triacylglycerol was seen in both serum as well as liver lipids in rats fed with SO [Tables -3.3 and 3.4]. This can be partly explained by the increased exogenous and endogenous fatty acid oxidation, which leads to the decreased incorporation of substrate (free fatty acid) for esterification. Fatty acid oxidation in mitochondria is more pronounced compared to peroxisomal β-oxidation. Hypolipidemic drugs such as fibrate and its related drugs function by enhancing peroxisomal β-oxidation which causes decreased ratio of β-hydroxybutyrate and acetoacetate which in turn decreases redox potential in the mitochondria. This causes enhanced ketone body production. Sesamin has been shown to be a peroxisomal proliferator like fibrate drug and causes marked increase in peroxisomal β-oxidation [Kohout et al, 1971; Goh and Heimberg, 1977; Guzuman and Geelan,

1993; Kushiro et al, 2004]. Hence in our study feeding SO may induce peroxisomal β-oxidation, which in turn leads to the stimulation of ketone body production. This may lead to the decreased conversion of fatty acid to triglyceride and its secretion.

The intake of dietary PUFA results in high PUFA in membrane lipids and enhances lipid peroxidation in blood and tissues, which is the prime factor in the onset of various disease conditions [Sanueni et al, 1983]. It has been showed that, sesame lignans enhance vitamin E levels when fed with diet containing low α-tocopherol content in rats facilitating in scavenging of free radicals and reactive oxygen species [Yamashita et al, 1992]. In the present study, the SO is compared with GNO for its protective role in iron induced toxic conditions. Feeding SO lowers the lipid peroxidation in serum and liver when compared to GNO fed group. The lipid peroxidation was brought down due to the presence of endogenous antioxidants in SO with good amounts of tocopherol. However, individual compounds were not separated and tested for inhibiting peroxidation. But whole SO showed to protect the damage to certain extent. Antioxidant enzymes activities in the liver were also enhanced due to feeding of SO and the mechanism for regulation of these increased activities of enzymes was not known. The beneficial effects of sesamin in liver damage caused by due to alcohol and carbon tetrachloride was rerported earlier [Akimoto et al, 1993].

The injection of iron enhanced the antioxidant enzyme level is in agreement with earlier results reported [Pulla Reddy and Lokesh, 1994]. The dietary sesamin at 2 g/kg inhibited lipid peroxidation in rats fed docosahexaenoic acid [Ikeda et al, 2003]. The beneficial effects of sesamin in liver damage caused due to alcohol and carbon tetrachloride was reported earlier [Akimoto et al, 1993]. Defatted sesame flour containing 1% sesaminol glucosides at 10% level in the diet decreased the susceptibility to oxidative stress in rabbits fed cholesterol due to the presence of antioxidative activity of sesaminol [Hwakang et al, 1999]. Sesame seed and its lignans known to produce marked enhancement of vitamin E activity in rats fed with low α-tocopherol diet [Yamashita et al, 1992]. They have also demonstrated that γ-tocopherol and sesame seed lignans act synergistically to produce vitamin E activity in rats. Sesamin enhances the tocopherol concentration by inhibiting tocopherol catabolism [Parker et al, 2000]. Antioxidant lignans from *Machilus thunbergii* which contains sesamin and other components were effective in protecting carbon tetrachloride injured primary cultures of rat hepatocytes [Yu et al, 2000].

Dietary sesamin could efficiently improve the abnormal vasodilator and vasoconstrictor responses in deoxycorticosterone acetate-salt induced hypertensive conditions (Matsumara et al, 1995]. Sesamin and tocopherol synergistically suppress lipid peroxides in rats fed high docosahexaenoic acid diet [Yamashita et al, 2000].

The serum enzymes (ALAT, ASAT and LDH) activities were increased in iron injected condition suggesting that the iron injection caused hepatic damage. Whereas feeding of sesame oil significantly decreased the activities of these enzymes indicating prevention of leakage of these enzymes from liver to serum. The antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase activities were increased in sesame oil fed group.

Inhibiting the cholesterol absorption from the intestine and cholesterol biosynthesis in the liver and increased excretion of bile acids and cholesterol in bile and feces resulted in the hypocholesterolemic effect of SO. The results also showed that sesame oil could successfully withstand lipid peroxidation in vivo by enhancing the activities antioxidant enzymes.

In conclusion feeding whole sesame oil has showed the health beneficial effects of isolated sesamin and episesamin, though it contains less amounts of sesamin.

Chapter – IV

Studies on the influence of spices and sesame oil on CGS induction in mice

Introduction

Bile plays an important role in the digestion of lipids. The main constituents of bile are; cholesterol, phospholipid, bile acids and minor amounts of proteins. Any disturbance in the composition of bile leads to several complications. Cholesterol gallstone (CGS) pathogenesis is one of the important disorders arising out of altered hepatic and biliary chlolesterol homeostasis. CGS disease is a multi-factorial disease involving both environmental as well as genetic factors [Johnston and Kaplan, 1993; Juvonen, 1994]. Gallstones are formed in the gallbladder due to precipitation of cholesterol, bilirubin and calcium salts in bile. The majority of gallstones are contributed by cholesterol and very small numbers of gallstones are primarily composed of calcium salts of bilirubin and phosphate. The pathologic conditions that generally precede the occurrence of CGS are: lithogenic bile, gallbladder stasis and short nucleation time. Lithogenicity of bile is determined by relative concentration of three main components viz; bile acids, phospholipids and cholesterol. Generally lithogenic bile occurs with disruption of cholesterol homeostasis, leading to increased cholesterol secretion and subsequent cholesterol supersaturation of bile [Apstein and Carey, 1996; Marzolo et al, 1990]. Gallbladder stasis increases the opportunity for concentration of supersaturated bile in the gallbladder to form gallstones. It is universally accepted that cholesterol supersaturation in bile is the pre-requisite step in the onset of cholesterol gallstones.

The spices – fenugreek, garlic, ginger, onion, red pepper and turmeric have been shown to be effective as hypocholesterolemic agents under conditions of experimentally induced hypercholesterolemia and hyperlipidemia. All the studies in which spices have been shown to influence cholesterol and /or triglyceride levels in blood and liver, lipoprotein cholesterl levels, fecal excretion of sterols and bile acids and biliary secretion of cholesterol and bile acids have been reviewed [Srinivasan et al, 2004]. Earlier it has been shown that curcumin and capsaicin at 0.5 and 0.015% level respectively inhibited the induction of cholesterol gallstones in mice [Hussain and Chandrasekara, 1993].

In the previous chapter, it has been demonstrated that sesame oil is a good

hypocholesterolemic and antioxidant agent. Spices (garlic and onion even though later is not strictly considered as spice but it is commonly used along with other spices) have been shown to be hypocholesterolemic and cholagoguic agents [Kamanna and Chandrasekhara, 1982; Babu and Srinivasan, 1997; Sambaiah and Srinivasan, 1991]. However there are no reports regarding the role of sesame oil and spices [garlic and onion] on induction of cholesterol gallstone in mice.

The objectives of the present study were: I) to induce CGS in experimental animals (mice) with lithogenic diet and ii) to study the influence of sesame oil and raw and heat processed garlic and onion in reducing or preventing incidence and severity of CGS.

Experimental protocol-

Standardization of the induction of cholesterol gallstones in mice:

Weanling male albino swiss mice weighing about 36 g were grouped on body weight basis into 6 groups of 4 animals each and fed with lithogenic diet (basal diet containing 0.5% cholesterol and 0.25% bile salts) over a period of 20 weeks to establish the induction of CGS in gallbladders of mice. One group of animals was sacrificed at the end of every 4 weeks of feeding after overnight fast. Body weight, organs weight and gallbladder weight were noted. CGS scoring was done as described earlier. Serum, liver and bile lipids were analyzed and cholesterol saturation index (CSI) of bile was calculated as described earlier.

Influence of of SO and spices on CGS induction

Influence of SO and raw and heat processed garlic and onion was studied in animals fed lithogenic diet. Weanling male albino swiss mice weighing 22 g were grouped into 7 groups of 18 animals each. They were fed with lithogenic diet containing 0.5% cholesterol and 0.25% bile salts and test diets: raw garlic (RG), heat processed garlic (HPG), raw onion (RO), heat processed onion (HPO) and sesame oil (SO) for 10 weeks. At the end of experimental period, animals were sacrificed after overnight fast. Body weight, organ weights and gallbladder weight were noted. CGS scoring was done as described earlier. Serum, liver and bile were analyzed for various constituents.

Results

Induction of CGS in mice

To evaluate the optimum time for the induction of CGS, mice were fed on the lithogenic diet over a period of time and the gallbladders were examined for the presence of gallstones. Results showed marginal increase in body weight and liver weight. Examination of gallbladders showed appearance of very fine crystals of cholesterol between 5 and 6 weeks of feeding and cholesterol crystals or stones bigger in size were seen between 8 - 10 weeks of feeding. The CGS induction was observed in 100 % of animals after feeding lithogenic diet for 10 weeks. During this period, the amount of gallbladder bile was also increased significantly [Table – 4.1]. Serum and liver cholesterol levels also showed a significant rise throughout the feeding period. Bile cholesterol saturation index (CSI) was 0.56 at the beginning and it was increased to 1.87 at 10 weeks of feeding lithogenic diet. The relationship

between serum, liver and bile cholesterol, CGS score and CSI during the induction of CGS is presented in Fig. 4.1. It shows that all the parameters were positively related to the development of cholesterol gallstones during induction period and reached maximum by 10 weeks of feeding and later did not change much. Therefore, it was considered that 10 weeks feeding would be optimum for the induction of CGS in mice.

Table 4.1: Influence of lithogenic diet on cholesterol gallstone induction in mice

Feeding period (Weeks)	Body wt. (g)	Liver wt.(g)	Bile vol. (μl)	CGS score
0	36.2 ± 1.08	1.60 ± 0.10	22.0 ± 2.30	-
4	42.0 ± 2.00	1.97 ± 0.20	26.3 ± 1.82	-
8	40.0 ± 1.02	2.32 ± 0.10	25.2 ± 3.20	++
10	41.0 ± 2.10	2.40 ± 0.50	30.1 ± 1.72	++++
16	36.0 ± 0.92	2.99 ± 0.20	32.6 ± 3.21	++++
20	40.0 ± 3.10	3.04 ± 0.34	38.9 ± 3.00	++++

Values are mean ± SD of 4 animals/group

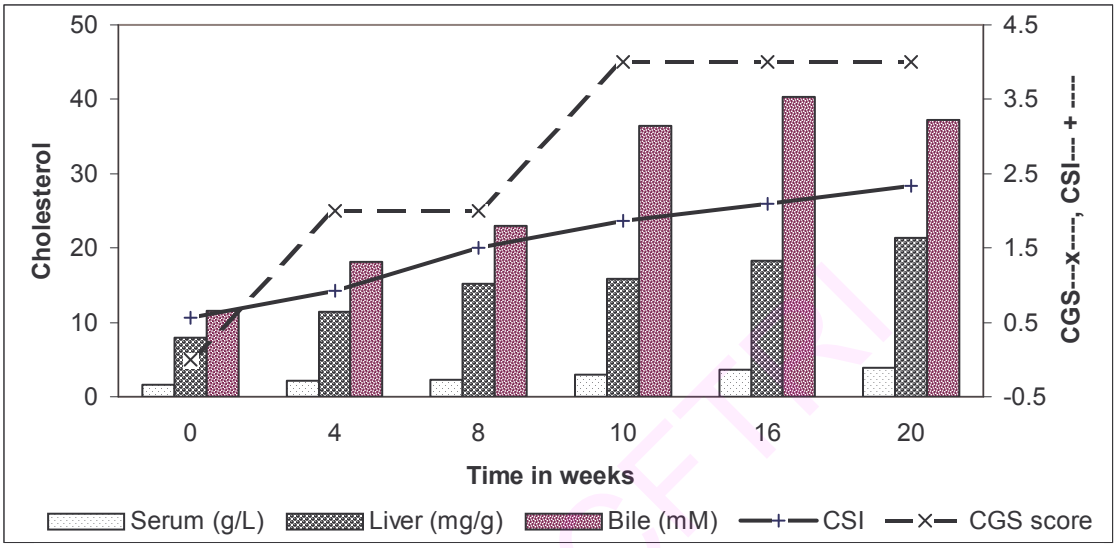


Fig – 4.1 Relationship between serum, liver, bile cholesterol and CGS Score and CSI of bile during induction of CGS in mice

Effect of spices and sesame oil on growth parameters during CGS induction

Effect of SO and spices (garlic and onion) on CGS induction in mice was carried out by feeding LG diet with or without SO, garlic or onion for a period of 10 weeks. During the induction of CGS, the body weight and organs weight of animals after feeding dietary garlic, onion (raw and heat processed) and SO are given in Table - 4.2. There were no significant differences in the total body weight of animals between the groups at the end of feeding period (10 weeks). The liver showed significant increase in weight in lithogenic diet fed group. On the other hand feeding of SO, HPO, RO, HPG and RG decreased the liver weight but it was not statistically significant. Kidney and heart weights were increased significantly upon LG diet feeding and spices and SO significantly reduced the increased weights. Spleen weight was not altered. The gallbladder size and weight were significantly increasedby LG diet and feeding of spices and SO significantly decreased both gallbladder size and its weight (Table – 4.3). Animals fed on control diet showed no

sign of any cholesterol crystal or stone in the gall bladder, whereas all the animals in LG diet group showed CGS (Table - 4.3 and Fig. 4.2). Animals fed on LG diet supplemented with spices and SO showed significant reduction in the incidence of CGS. The % incidence of CGS occurred was calculated by taking the incidence in LG diet as 100 %. The lowest incidence of CGS [61.2%] was occurred in the HPO group. In other words the reduction in the CGS in experimental animals ranged from 14.5 – 38.8 % showing that both spices [raw and heat processed] and SO were effective in reducing the formation of CGS.

Table 4.2: Effect of spices and SO on body weight and organ weights in mice

Dietary group	B.wt. (g)	Liver (g)	Kidney (mg)	Heart (mg)	Spleen (mg)
Control	41.4±5.82	1.61±0.39	411.8±8.57	136.0±3.82	105.7±8.27
LG	46.9±3.03[a]	2.90±0.19[a]	518.7±17.56[a]	158.5±6.27[a]	106.9±4.94
LG+RG	41.7±1.56	2.62±0.19	427.2±5.04[b]	136.5±3.67[b]	100.0±3.55
LG+HPG	43.0±3.77	2.74±0.19	401.8±4.31[b]	149.3±1.46	105.0±4.48
LG+RO	39.5±3.52	2.54±0.30	338.9±5.85[b]	122.1±3.99[b]	91.7±4.18
LG+HPO	43.3±1.93	2.55±0.14	384.8±2.30[b]	142.9±1.98[b]	96.1±1.56
LG+SO	44.4±2.13	2.46±0.21	396.5±3.13[b]	144.6±2.19[b]	102.3±3.26

Values are mean ± SD of 18 mice /group.

[a] Statistically significant when compared to control group at P <0.01

[b] Statistically significant when compared to LG group at P <0.01

RG – raw garlic, HPG – heat processed garlic, RO – raw onion,

HPO – heat processed onion, SO – sesame oil

Table 4.3: Effect of spices and SO on gallbladder weight and cholesterol gallstones (CGS) incidence in mice

Dietary groups	Gallbladder wt (mg)	CGS score	CGS incidence (%)	CGS reduction (%)
Control	25.5 ± 4.87	0.00	0.0	-
LG	67.3 ± 4.38[a]	3.40 ± 0.80[a]	100.0	-
LG+RG	43.3 ± 2.53[b]	3.05 ± 0.84[b]	73.2	26.7
LG+HPG	38.5 ± 3.55[b]	3.02 ± 0.71[b]	85.5	14.5
LG+RO	36.9 ± 3.99[b]	2.33 ± 1.15[b]	75.2	24.7
LG+HPO	30.0 ± 1.75[b]	1.85 ± 1.27[b]	61.2	38.8
LG+SO	36.5 ± 1.93[b]	2.98 ± 0.65[b]	74.3	25.6

Values are mean ± SD of 18 mice/group.

[a] Statistically significant when compared to control group at $P < 0.01$

[b] Statistically significant when compared to LG group at $P < 0.01$

Group legends are as in Table - 4.2

CGS score – Values are average of 18 animals/group. Scored by 4 different individuals grading was done on a 4 point scale (0-4).

Liver with GB from control mice

Liver with GB from lithogenic mice

Control

LG+RG

LG

LG+HPG

LG+RO

LG+SO

LG+HPO

Fig 4.2 Effect of spices and SO on gallbladder size and cholesterol gallstones (CGS) induction in mice

Group legends are as in Table - 4.2

Effect of spices and sesame oil on serum lipids during CGS induction

The serum lipids serve as a marker for determining various pathological conditions related to lipid metabolism in the body. Hence the serum lipids were estimated and the results are presented in Table- 4.4. The LG diet feeding caused hypercholesterolemia and serum cholesterol increased by 2.4 times compared to control animals. Feeding of spices or SO with LG diet prevented the raise in serum cholesterol levels by 33, 29, 31, 34 and 30 % in RG, HPG, RO, HPO and SO groups respectively. The phospholipid content of serum was significantly reduced by LG diet, whereas feeding of spices and SO significantly prevented this decrease in phospholipid content. The triglyceride content was significantly higher in LG group compared to control group. Feeding of SO and HPO reduced triglycerides significantly but the reduction was not significant in other groups. Feeding of LG diet significantly increased the C/PL ratio and spices and SO significantly reduced the increased C/PL ratio.

Effect of spices and sesame oil on liver lipids during CGS induction

Liver is the major site for lipid metabolism and is also sensitive to dietary lipids. Liver lipids were estimated and the results are given in Table-4.5. The total cholesterol content was increased in LG diet group by 2.8 folds compared to control group. Feeding of spices and SO significantly decreased the total cholesterol content by 25, 15, 15, 30 and 15 % in RG, HPG, RO, HPO and SO groups respectively compared to LG diet group. The phospholipid content was decreased in LG diet group compared to control group and it was increased in all experimental groups. The increased C/PL ratio was brought down significantly by spices and SO feeding. Feeding of LG diet increased TG content of liver and inclusion of spices and SO in the diet significantly decreased the triglyceride content.

Effect of spices and SO on biliary lipid profile during CGS induction

Bile is the major route through which cholesterol and its metabolite products [bile acids] are excreted. It also plays an important role in the digestion and absorption of fat in the intestine. Biliary lipid composition is important in keeping the bile physiologically active. The biliary lipid parameters were estimated and the results are given in Table - 4.6. LG diet increased the cholesterol content of bile by 6 times. Spices and SO reduced the biliary cholesterol content significantly. The reduction ranged from 35 - 66 % in various experimental groups compared to LG diet group.

Table 4.4: Effect of spices and SO on serum lipid profile during CGS induction in mice

Groups	Total cholesterol	Phospholipid	Triglycerides	C/PL
	(mg/dL)			
Control	185.5±23.05	315.5±14.22	149.5±9.19	0.58±0.07
LG	453.9±32.04[a]	160.5±15.12[a]	185.4±11.25[a]	2.84±0.29[a]
LG+RG	304.8±28.10[b]	284.0±13.57[b]	172.0±9.39	1.07±0.11[b]
LG+HPG	323.4±17.80[b]	279.9±14.76[b]	177.2±6.95	1.80±0.17[b]
LG+RO	314.3±17.77[b]	211.8±13.39[b]	173.7±6.74	1.48±0.12[b]
LG+HPO	298.8±19.4[b]	287.0±18.56[b]	165.9±10.62[b]	1.04±0.08[b]
LG+SO	319.2±16.24[b]	279.1±14.56[b]	162.3±6.95[b]	1.14±0.12[b]

Values are mean ± SD of 6 samples/group, each sample constituting 3 mice

[a] Statistically significant when compared to control group at $P < 0.01$

[b] Statistically significant when compared to LG group at $P < 0.01$

Group legends are as in Table –4.2

C/PL - cholesterol/phospholipid ratio

Table 4.5: Effect of spices and SO on liver lipid profile during CGS induction in mice

Groups	Total cholesterol	Phospholipid	Triglycerides	C/PL
	(mg/g)			
Control	8.63±1.51	42.74±2.29	27.04±4.01	0.20±0.04
LG	21.25±2.07 [a]	30.61±2.66[a]	53.66±2.72[a]	0.69±0.15[a]
LG+RG	16.04±2.07[b]	39.23±2.10[b]	43.51±2.84[b]	0.41±0.10[b]
LG+HPG	18.07±1.15[b]	34.67±2.65[b]	47.46±2.15[b]	0.52±0.14[b]
LG+RO	18.01±2.04[b]	33.11±2.04[b]	46.33±1.72[b]	0.54±0.09[b]
LG+HPO	14.89±2.40[b]	42.72±2.69[b]	34.79±3.19[b]	0.34±0.07[b]
LG+SO	18.04±2.93[b]	38.21±2.85[b]	42.31±2.09[b]	0.47±0.11[b]

Values are mean ± SD of 6 samples/group, each sample constituting 3 mice

[a] Statistically significant when compared to control group at $P < 0.01$

[b] Statistically significant when compared to LG group at $P < 0.01$

Group legends are as in Table –4.2

Table 4.6: Effect of spices and SO on biliary lipid profile during CGS induction in mice

Dietary group	Cholesterol	Phospholipid	Bile acids	Total lipid (g/dL)
	mM			
Control	5.56±0.65	12.33±1.79	216.4±25.06	10.91±1.29
LG	33.46±3.30[a]	21.15±3.24[a]	228.0±15.17	11.44±0.95
LG+RG	13.70±2.98[b]	18.50±3.84	218.5±19.17	11.10±1.24
LG+HPG	21.70±4.01[b]	22.52±3.60	219.2±15.94	11.17±1.18
LG+RO	17.03±3.60[b]	20.19±3.95	215.3±20.53	10.96±1.17
LG+HPO	11.53±1.95[b]	15.73±3.13[b]	222.6±19.79	11.25±0.84
LG+SO	14.70±1.61[b]	19.61±2.71	210.7±11.31	11.23±1.02

Values are mean ± SD of 6 samples/group, each sample constituting 3 mice

[a] Statistically significant when compared to control group at $P < 0.01$

[b] Statistically significant when compared to LG group at $P < 0.01$

Group legends are as in Table – 4.2

The phospholipid is one of the major constituents of bile, which helps the bile to be physiologically active. Any disturbance or alteration in the phospholipid and bile acidst results in the precipitation of cholesterol in bile. The phospholipid content was increased in the LG group by 72 % compared to control. On the other hand, RG, HPO and SO feeding decreased the phospholipid content by 13, 26, and 7 % respectively compared to LG group. In other two groups (HPG and RO) there was a marginal increase in phospholipid content. Bile acids (salts) form a major part of the bile solids content. They are synthesized from the parent molecule cholesterol. The majority of the cholesterol is excreted from the system after converting into bile acids.

Increase in the bile acid synthesis and excretion in bile tends to decrease the cholesterol content and lithogenicity of bile. The bile acid content in the bile was increased in the LG group by 6% compared to control. But feeding of RG, HPG, RO, HPO and SO did not alter the bile acid content in bile.

When all the three constituents of bile (cholesterol, phospholipids and bile acids) represented in a pie - diagram as shown in Figure - 4.3, It shows that in control group, cholesterol represents 2 %, phospholipids 9 % and bile acids 89 %. This composition was altered by LG diet to 11% cholesterol, 14 % phospholipid and 75 % bile acids. Feeding of spices or SO increased the relative proportion of bile acid and phospholipid content and decreased the cholesterol content in the bile. The decrease was 59, 35, 49, 66 and 56% in cholesterol content in RG, HPG, RO, HPO, and SO groups respectively compared to LG diet fed group.

In order to evaluate the relevance of the bile lipid profile in CGS formation, C/PL and C/BA ratios in bile were calculated. The higher ratios of C/PL and C/BS are the indicators of lithogenic bile and it is very important to keep the cholesterol in soluble form. Hence the ratios of biliary components were tabulated and given in Table - 4.7. The C/PL ratio was 1.58 in LG diet fed animals compared to 0.45 in control group. The C/PL ratio was brought down to 0.74, 0.96, 0.84, 0.73 and 0.75 in RG, HPG, RO, HPO and SO groups respectively. The C/BA ratio was 0.15 in LG diet fed animals compared to 0.03 in control group. The C/BA ratio was brought down to 0.06, 0.10, 0.08, 0.05 and 0.07 in RG, HPG, RO, HPO and SO groups respectively.

The cholesterol saturation index [CSI] of the bile is a measure of supersaturation of bile with cholesterol. It was significantly higher in LG group [1.90] and feeding of spices and SO brought it down by 55, 43, 34, 52 and 49 % in HPO, RO, HPG, RG and SO groups respectively. These results are given in Table - 4.8.

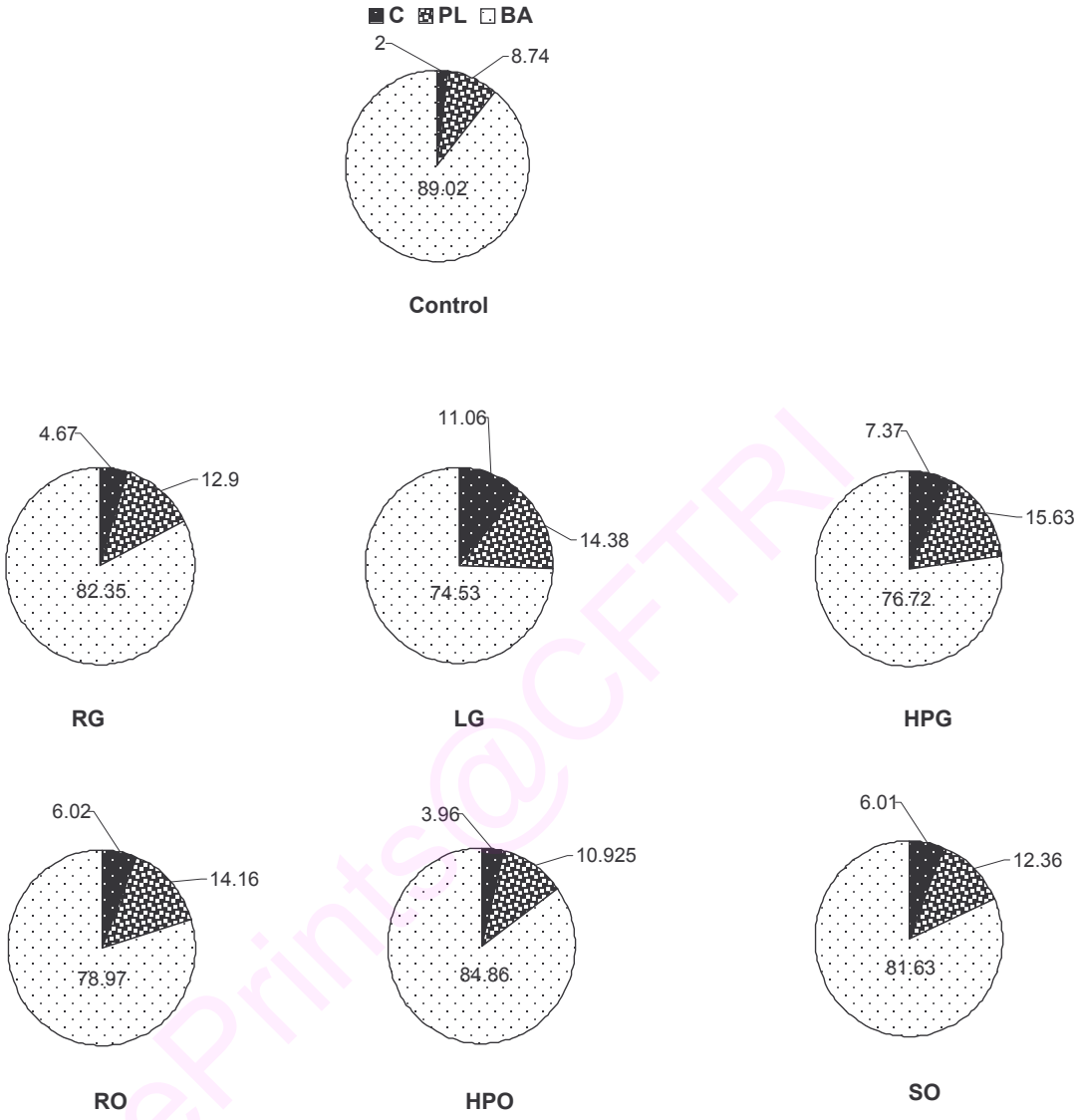


Fig 4.3: Pie-diagrams showing relative % composition of cholesterol, phospholipids and bile acids in biliary lipid in control, lithogenic and spices and SO fed animals

Table 4.7: Effect of spices and SO on C/PL and C/BA ratio in bile

Dietary group	C/PL	C/BA
Control	0.45	0.03
LG	1.58	0.15
LG+RG	0.74	0.06
LG+HPG	0.96	0.10
LG+RO	0.84	0.08
LG+HPO	0.73	0.05
LG+SO	0.75	0.07

Values are mean of 6 samples/group, each sample constituting 3 mice

C/PL - cholesterol/phospholipid

C/BA - cholesterol/bile acids

Group legends are as in Table – 4.2

The bile acids are natural detergents that solubilize lipids in the intestinal tract and in bile. The relative balance of hydrophillic and hydrophobic bile acids in the bile depends upon their state of ionization, number, position and orientation of hydroxyl groups, and by the presence and nature of ring or side chain esters. The bile acids taurocholic (TC), tauromuricholic (TMC) and tauroursodeoxychoate (TUDC) were termed as hydrophilic bile salts. The taurochenodeoxy cholic acid (TCDC), and taurodeoxycholic acid (TDC) were termed as hydrophobic bile acids [Heuman et al, 1989].

The hydrophobic index (HI) is the net sum of hydrophobic and hydrophilic bile acids present in the bile. The hydropphobicity index (HI) was –0.145 in the control bile and it was increased to +0.054 in the LG bile. The RG, HPG, RO, HPO and SO feeding decreased the HI of bile to –0.079, -0.032, -0.073, -0.088 and –0.074 respectively and is given in Table - 4.8.

The mole % of bile acids present in the gallbladder bile of mice fed LG diet and test diets are given in Table - 4.9. In the LG group, the taurocholic acid (TC) content was increased compared to the control group. The hydrophobic bile acids such as taurochenodeoxycholic acid (TCDC), and taurodeoxycholic acid (TDC) content were increased and the hydrophilic bile acids such as tauromuricholic acid (TMC) and tauroursodeoxycholic acid (TUDC) were decreased in LG diet fed group. On the other hand feeding spices and SO increased the content of TMC and TUDC (hydrophilic) and decreased the TCDC and TDC content (hydrophobic) compared to LG diet group.

Table 4.8: Effect of spices and SO on CSI and HI of bile during CGS induction in mice

Dietary group	CSI	HI
Control	0.51± 0.07	-0.145 ± 0.05
LG	1.90± 0.25[a]	+0.054 ± 0.02[a]
LG+RG	0.92± 0.13[b]	-0.079 ± 0.03[b]
LG+HPG	1.25± 0.13[b]	-0.032 ± 0.02[b]
LG+RO	1.09± 0.13[b]	-0.073 ± 0.008[b]
LG+HPO	0.86± 0.16[b]	-0.088 ± 0.021[b]
LG+SO	0.96± 0.11[b]	-0.074 ± 0.03[b]

Values are mean ± SD of 6 samples/group, each sample constituting 3 mice

[a] Statistically significant when compared to control group at $P < 0.01$.

[b] Statistically significant when compared to LG group at $P < 0.01$.

Group legends are as in table – 4.2,

CSI-Cholesterol saturation index,

HI-Hydrophobic index

Biliary fatty acid composition reflects the type of fatty acids present in the phospholipids and cholesterol esters. Dietary lipids play a vital role in determining the type of fatty acids present in the phospholipids. Hence biliary fatty acids present in phospholipids was analysed and given in Table - 4.10. Oleic acid was significantly decreased whereas palmitic and linoleic acids were increased in LG group compared to controls. By feeding HPO, RO and HPG the oleic acid content was increased by 10,18 and 20 respectively compared to LG diet group. SO did not have any effect on the content of oleic acid. The linoleic acid content was not altered in the spice and SO fed groups.

Effect of spices and sesame oil on enzymes of cholesterol metabolism during CGS induction

The HMG-CoA reductase is the key regulatory enzyme involved in the biosynthesis of cholesterol and cholesterol 7$\propto$-hydroxylase and sterol 27-hydroxylase are involved in the biosynthesis of bile acids from cholesterol. The cholesterol 7α - hydroxylase and sterol 27 - hydroxylase enzymes regulate the bile acid biosynthesis in liver. Spices and dietary lipids are known to play a pivotal role in the metabolism of cholesterol. Hence, the activities of these enzymes were estimated and results are presented in Table - 4.11. Feeding of LG diet inhibited the HMG-CoA reductase activity significantly. On the other hand enzyme activity was increased by 92, 44, 61, 35 and 71% in HPO, RO, HPG, RG and SO fed groups respectively compared to LG group. Cholesterol 7α - hydroxylase and sterol 27-hydroxylase activities were inhibited significantly by LG diet compared to control group. The spices and SO feeding enhanced the activity of both enzymes. Cholesterol 7α - hydroxylase activity was increased by 59, 25, 18, 37 and 33 % in HPO, RO, HPG, RG and SO fed groups respectively compared to LG group. Similarly, sterol 27-hydroxylase activity was increased in HPO, RO, HPG, RG and SO fed groups by 87, 54, 42, 80 and 74 % respectively when compared to LG group.

Relationship between serum, liver and bile cholesterol level and CSI and CGS score and effect of spices and SO

Cholesterol level in serum, liver and bile serves as marker in CGS pathogenesis and plays an important role in the formation of CGS. Relationship between serum, liver and bile cholesterol and CGS and CSI during induction of CGS is presented in

Fig. 4.4, 4.5 and 4.6 respectively. These figures summarize the results presented in various tables and figures presented in this chapter. Cholesterol level in serum, liver and bile was significantly increased in LG diet group compared to control group. Feeding of spices or SO were significantly reduced the cholesterol in all. The cholesterol saturation index (CSI) and CGS score were well compared with the serum, liver and biliary cholesterol content in the groups fed lithogenic diet. This directly suggests a strong relationship between cholesterol in the body and cholesterol saturation index (CSI) and gallstone pathogenesis.

Table 4.11: Effect of spices and SO on hepatic cholesterol metabolizing enzymes in mice

Dietary groups	HMG-CoA reductase*	Cholesterol 7α-hydroxylase**	Sterol 27-hydroxylase**
Control	67.35 $\pm$ 43.86	14.61 $\pm$1.96	48.94 $\pm$ 5.11
LG	28.64 $\pm$ 4.31[a]	3.86 $\pm$ 0.73[a]	16.36 $\pm$ 2.13[a]
LG+RG	38.71 $\pm$ 2.81[b]	5.30 $\pm$ 1.20[b]	29.41 $\pm$ 3.18[b]
LG+HPG	46.19 $\pm$ 5.63[b]	4.56 $\pm$ 0.81[b]	23.26 $\pm$ 4.73[b]
LG+RO	41.32 $\pm$ 2.98[b]	4.82 $\pm$ 1.12[b]	25.18 $\pm$ 3.88[b]
LG+HPO	54.86 $\pm$ 6.78[b]	6.14 $\pm$ 0.64[b]	30.61 $\pm$ 2.98[b]
LG+SO	50.23 $\pm$ 3.21[b]	5.12 $\pm$ 0.31[b]	28.54 $\pm$ 3.12[b]

Values are mean $\pm$ SD of 5 samples,

*nmoles of CoA formed /min/mg protein, **pmoles of 7α-hydroxycholesterol and 27-hydroxycholesterol formed /min/mg protein.

[a] Statistically significant when compared to control group at $P < 0.01$.

[b] Statistically significant when compared to LG group at $P < 0.01$.

Group legends are as in Table –4.2

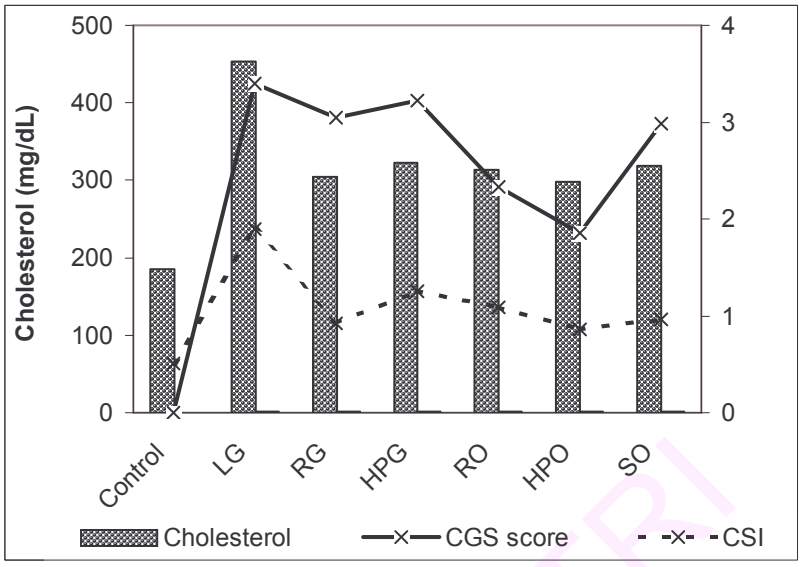


Fig 4.4: Relationship between serum cholesterol, CGS Score and CSI of bile during induction of CGS in mice

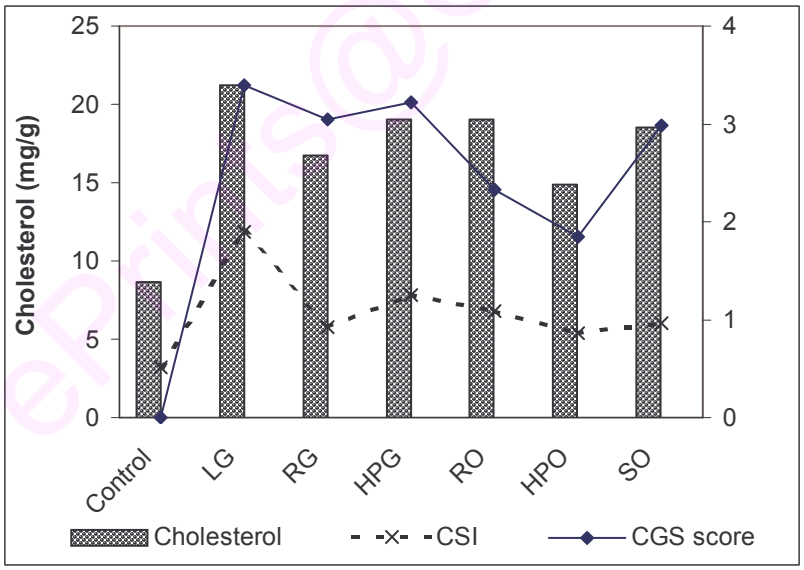


Fig 4.5: Relationship between liver cholesterol, CGS Score and CSI of bile during induction of CGS in mice

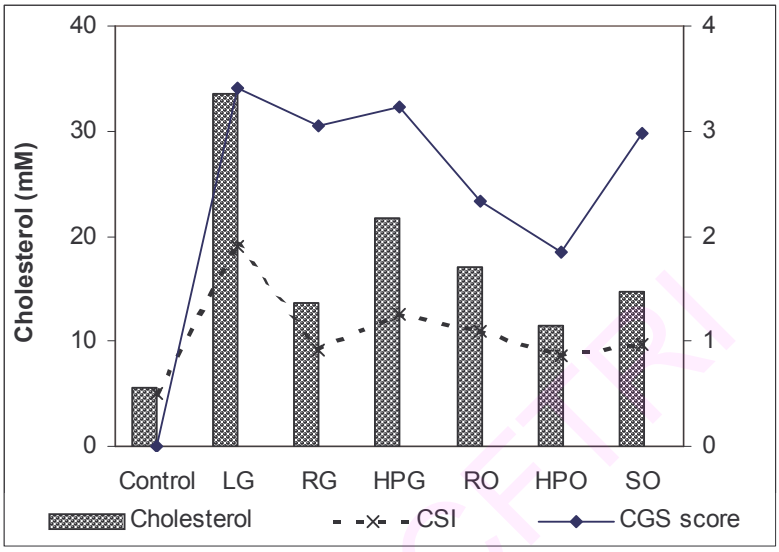


Fig 4.6: Relationship between bile cholesterol, CGS Score and CSI of bile during induction of CGS in mice

Relationship between CSI and CGS score and cholesterol degrading enzymes and effect of spices and SO

The cholesterol saturation index was compared with cholesterol degrading enzymes in various groups. LG feeding increased the CSI and decreased the C7h and S25h activities (Fig 4.7).

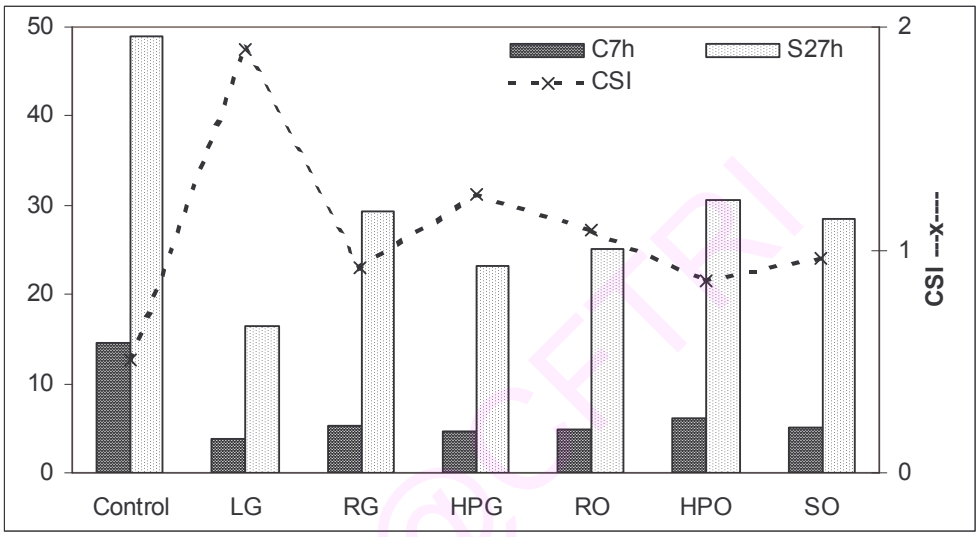


Fig 4.7: Relationship between CSI of bile and cholesterol degrading enzymes during CGS induction in mice

Values are expressed in pmol/min/mg protein (C7h, S27h).

Abbreviations: C7h-cholesterol 7α-hydroxylase,

S27h-Sterol 27-hydroxylase

CSI – cholesterol saturation index

Discussion

The present study used the well-studied mouse model for induction of CGS by feeding lithogenic (LG) diet [Tepperman et al, 1964]. Results showed that optimum duration of LG diet feeding for CGS induction in 100 % of animals was 10 weeks.

Spices have been shown to possess hypolipidemic, hypocholesterolemic and hydrocholagougic effects [Srinivasan et al, 2004]. It has been reported that spices and their active priciples reduced biliary cholesterol and increased the bile acid concentration and water content in the bile [Bhat et al, 1984, 1985; Sambaiah and Satyanarayana, 1980; Sambaiah and Srinivasan, 1991]. The net result of these effects would result in dilution of bile. Dilute biles have prolonged nucleation times [Van-Erpecum and Henegouwen, 1989]. Previously curcumin and capsaicin (coloring principle of turmeric and pungent principle of red pepper respectively) have been shown to prevent cholesterol gallstone (CGS) formation in mice [Hussain and Chandresekhara 1993; 1994a]. We have selected garlic and onion which are commonly consumed spices and known to possess hypolipidemic and hydrocholagougic effects. Cooking or roasting alters the nature of many food constituents such as starches and proteins by changing their physical, chemical and nutritional properties. It also changes the bioavailability of proteins, carbohydrates, lipids and vitamins. Normally spices are consumed as such in their natural form or in the form of spice mixture or curry powder. In Indian homes they are added to curries and subjected to cooking before they are consumed. It has been reported earlier that boiling of spice mixes with food ingredients results in loss of active principles of common spices during domestic cooking [Srinivasan et al, 1992]. Earlier studies on hypolipidemic effect of spices were carried out with raw spices without under going any processing conditions such as boiling, heating or frying. In the present study, the effect of boiling (spice powder was boiled for 15 min and cooled) on the anti-lithogenic potential was studied. The boiled spice powders were mixed with AIN-76 basal diet and pellets were prepared and used. This study was under taken to establish whether boiling of spices have similar anti-lithogenic properties or not compared to raw spices. These studies were not intended to isolate, identify and characterize the degraded or modified compounds formed if any during heat processing and their effects individually. In addition to garlic and onion, we have also continued our studies with sesame oil (SO), which showed hypolipidemic effect and affected bile secretion and composition (chapter – III). The goals of the present study

were to study the influence of garlic and onion (raw and heat processed) and sesame oil on cholesterol gallstone formation.

Feeding of LG diet did not influence body weight but significantly increased liver weight due to fat deposition (Fig - 4.2) and high cholesterol content (Table - 4.5). Feeding of SO, garlic and onion (raw and heat processed) did not cause any adverse effect on body weight and other organs except liver where it was slightly reduced. Feeding of LG diet to mice caused secretion of more cholesterol molecules into bile leading to the formation of lithogenic bile. LG diet feeding for 10 weeks induced CGS formation in all mice and it was consistent. Inclusion of raw / heat processed garlic (0.6 %), onion (2 %) or SO (5 %) in the diet significantly reduced the formation of CGS. The pathophysiological conditions that might be responsible for the cholesterol crystallization and gallstone formation is increased cholesterol content and decreased bile acids and phospholipids in bile. Wang et al, [1999] showed that gallstone susceptible mice secrete more cholesterol molecules into bile at canalicular membrane level leading to the formation of lithogenic bile. This leads to higher CSI resulting in the nucleation of cholesterol leading to the formation of CGS. The LG diet feeding increased the hepatic cholesterol (Table-4.5), a significant part of which is secreted into bile. This is accompanied by reduced bile acid and phospholipid secretion.

The supersaturation of bile with cholesterol (CSI >1) leads to the formation of CGS [Carey and Small, 1978]. The CSI of experimental groups is < 1 except in HPG and RO groups where it is only marginally >1 indicating that the cholesterol content of bile is low and bile acids and phospholipids are more compared to LG diet fed group. The inclusion of spices or SO in the diet prevented the CGS formation and is evident where the CSI is less than 1 (Table – 4.8). The CSI of the spices or SO fed animals was much lower than that of the LG group which means that, the bile of these animals has capacity to solubilise more cholesterol due to higher bile acids and phospholipids concentration present in it relative to cholesterol concentration. These results also indicated that CGS are also present in mice when the CSI is <1 and the effect of these spices and SO is only partial (Table – 4.3). It has been shown that CSI being >1 is a pre-requisite for the precipitation of cholesterol and subsequent stone formation but it is not always true as in some cases CGS were observed when CSI is < 1. This is because there may be several other factors that are responsible for the formation of CGS. Cholesterol secretion into bile is tightly coupled to phospholipid

and bile acid secretion [Robins et al, 1991].

Biliary C/PL secretion rates are considered to be closely linked to bile acid secretion rates and to be enhanced by bile acids. The C/PL ratios of bile indicate C/PL ratios of the vesicles whose secretion is induced by bile acids at the canaliculus [Wang et al, 1999]. In the present study during LG diet feeding there was a significant increase in C/PL ratio which paralleled the elevated CSI in bile. In contrast, experimental animals maintained on spice/SO diets showed a significant decrease in C/PL and CSI compared to LG diet group (Table-4.7 and 4.8). This shows that LG diet fed mice secrete more cholesterol molecules into bile at canalicular membrane level which is a major factor leading to the formation of lithogenic bile. Our results are also in agreement with Hoffmann and Grundy [1982] who observed that there was a high C/PL ratio in the bile of CGS patients. This suggests that in CGS patients as well as in mice nascent phospholipid vesicles at the canalicular level may be enriched with cholesteol and spices and SO preventing this enrichment.

The C/PL and C/BA ratios were significantly increased upon feeding LG diet indicating increased cholesterol in bile. Feeding of spices and SO reduced the C/PL and C/BA ratios significantly compared to LG diet group (Table - 4.7). Bile acids provide primary stimulus for bile flow and facilitate the secretion of lipids into bile [Booker et al, 1990; Tierney et al, 1993; Knox et al, 1991]. Biliary secretion of phospholipids is dependent on the bile acids. During bile formation, bile acids stimulate secretion of phospholipids from hepatocytes [Cohen et al, 1990]. Increased secretion of cholesterol is related to phospholipids with more hydrophobic molecular species of phospholipids in the bile [Robins et al, 1991]. Compared to control group, the total bile acid content of bile in LG group was increased slightly whereas the cholesterol content increased by 5 folds (Table - 4.6). On the other hand, feeding of spices and SO significantly decreased the biliary cholesterol and increased the bile acids though slightly making bile more hydrophilic. The phospholipid content was also increased relatively compared to the content of cholesterol in LG group and in spice and SO fed animals. The C/PL ratio was significantly increased in LG group, but feeding spices and SO decreased the C/PL ratio (Table - 4.6 and 4.7). Increases in the bile acids, bile flow and phospholipids help in solubilization of cholesterol in mixed micelles and vesicles the major carriers of cholesterol in bile. This stabilized the bile and increased the solubilization of cholesterol and prolonged the nucleation time of bile. It has been shown that in the gallbladder due to concentration of bile by

absorption of water, elevation of biliary vesicular C/PL ratio occurs leading to vesicular aggregation, which precedes nucleation [Van-Erpecum and Henegouwen, 1989; Roslyn et al, 1986]. As the bile gets concentrated in the gallbladder, the vesicles become rich in cholesterol. As a result the C/PL ratio of vesicles increases. The vesicles become unstable due to high C/PL ratio and nucleate cholesterol [Afdhal and Smith, 1990].

The hepato-biliary pathway is the major route for the removal of cholesterol from the body either after converting into bile acids or in the form of free cholesterol. Dietary cholesterol inhibits hepatic cholesterol synthesis, which in turn impairs bile acid synthesis. These three factors lead to supersaturation of bile with cholesterol (CSI >1). Bile with higher CSI (>1) is prone to cholesterol crystal nucleation. Hence biliary lipid secretion plays an important role in cholesterol homeostasis. Spices and SO feeding significantly increased the hydrophilic bile acids such as TC, TMC and TUDC (Table - 4.9). Earlier it has been reported that increased content of biliary deoxycholic acid in CGS patients or increased deoxycholic acid content in bile promotes rapid cholesterol crystallization and formation of CGS. In fact, it was demonstrated that deoxycholic acid is highly hydrophobic and was associated with human cholelithiasis [Reihner et al, 1991]. The hydrophobicity of bile acids plays an important role in gallstone formation. It was suggested that glycine conjugated bile acids are more hydrophobic which are lithogenic in nature, while taurine conjugates have opposite effects [Trautwein et al, 1993: Heuman et al, 1989: Armstrong and Carey, 1982]. It was reported that bile acids are conjugated with taurine in rodents [Haselwood and Wooton, 1950]. Reports show that taurine synthesis indirectly increased cholesterol 7α-hydroxylase activity thereby increases the synthesis of bile acids [Hajri et al, 1998]. The fatty acid composition of phospholipid exhibited significant influence on CSI in monkeys and humans. Berr et al, [1992] have noticed a negative relationship with linoleic acid. In the present study the linoleic acid and oleic acid content were increased to different extents by feeding spices and SO compared to LG diet.

The biosynthesis of cholesterol is regulated by HMG-CoA reductase activity whereas, biodegradation of cholesterol to bile acids in hepatocytes is carried by cholesterol 7$\propto$-hydroxylase and sterol-27-hydroxylase [Dietschy et al, 1993]. The HMG-CoA reductase activity was completely inhibited by LG diet due to negative feedback inhibition. Feeding spices and SO could restore the activity significantly. The

possible explanation for this can be that cholesterol synthesized by *de novo* process might be preferred over the exogenous cholesterol for conversion into bile acids and for further excretion in bile. Our results are not in concurrence with earlier reports where in HMG-CoA reductase activity was higher in CGS prevailing conditions in humans [Schoenfield, 1976]. On the other hand, Carulli et al, [1984] reported no changes in the HMG-CoA reductase activity during prevalence of CGS in humans. Similarly, cholesterol 7α-hydroxylase and sterol 27-hydroxylase activity were inhibited by feeding LG diet. Spices and SO increased the enzyme activity significantly. Earlier Srinivasan and Sambaiah [1991], showed that cholesterol 7α - hydroxylase activity in rats was increased by feeding spices.

The increase in the serum and liver cholesterol levels was reflected in the increased CSI in bile and CGS occurrence in LG diet fed animals. Feeding spices and SO significantly brought down the cholesterol content in serum and liver and it was evident in the lower CSI values and decreased CGS occurrence. The increased C/PL ratios in serum and liver reflected the higher CSI and CGS occurrence. The experimental diets significantly brought down the C/PL ratio, which reflected in the lower CSI and CGS occurrence. The lowered levels of cholesterol may be due to the combined result of decreased synthesis (as reflected in decreased HMG-CoA reductase activity) and increased conversion to bile acids (as reflected in increased cholesterol 7α- hydroxylase and sterol 27-hydroxylase activity).

The present study clearly demonstrated that feeding of raw and heat processed spices (garlic and onion) and SO inhibited the formation of CGS in mice by decreasing the cholesterol content in serum, liver and bile. This is also reflected in the lowering of CSI and CGS occurrence in mice. The dosage of garlic (0.6%) and onion (2%) included in the diet for experimental animals were 5 times higher than what is normally consumed regularly [Platel and Srinivasan, 2000] and this needs to be taken into consideration before extrapolating the results. Consumption of spices over a period of 10 weeks has an added benefit and no deleterious effect.

The present study showed that heat processing of onion (boiling for 15 min) was more effective than raw onion in alleviating the incidence of CGS in mice. On the other hand, heat processing of garlic results in the reduction of antilithogenic activity compared to raw garlic.

Chapter – V

Studies on the influence of spices and sesame oil on preformed CGS in mice

Introduction

The onset of cholesterol gallstone is associated with various physico-chemical disturbances in bile. It includes excess secretion of cholesterol and reduced secretion of bile acids by the liver into bile and increased absorption of water from the gallbladder thus creating an hydrophobic environment. These series of events lead to supersaturation of bile with cholesterol, a pre-requisite for CGS formation [Van-Erpecum and Van-Berge Henegouven, 1989].

The cholesterol is highly insoluble and in bile it is transported in vesicles and micelles. As the bile becomes supersaturated, vesicles become richer in cholesterol [Holzbach, 1990; Roslyn et al, 1986]. Nucleation of cholesterol takes place from cholesterol rich vesicles rather than micelles. The nucleated cholesterol crystals grow to form large crystals and finally resulting in the formation of CGS in gallbladder [Halpern et al, 1986b].

Earlier numerous studies using animal models have been carried out to evaluate the role of dietary components in preventing the cholesterol gallstones [Hayes et al, 1992]. It has been shown that dietary components like proteins [Hayes et al, 1992], carbohydrates [Thorton et al, 1983b], fibre [Ebihara and Kiriyama, 1985], fat [Mott et al, 1992] etc. play a role in the induction of cholesterol gallstones. On the other hand role of dietary factors in the regression or amelioration of already existing CGS is not studied extensively.

Generally, CGS treatment involves lithotripsy, cholecystectomy (surgical removal of gall bladder) or by drugs such as ursodexoycholate. But these treatments are having their own drawbacks and limitations and the chances of reoccurrence of stones are very much likely [Bouchier, 1990].

Hence, in this context an alternative and suitable non-invasive and safe treatment might be dietary intervention to regress the already existing CGS. In the previous chapter it has been clearly shown that spices (garlic and onion) and SO effectively inhibited the formation of CGS in mice. In this context, the present study was focused on the role of spices and SO on reduction of existing CGS in mice by feeding control diet with or without spices or SO for two time intervals of 5 and 10 weeks.

Experimental

Male swiss albino mice (262) were initially fed with lithogenic diet for 10 weeks and cholesterol gallstones were induced as described earlier (chapter IV). At the end of the CGS induction period, 10 animals were randomly selected and sacrificed to confirm the formation of CGS. All the procedures for the confimation of the presence of CGS were carried out as described previously. Remaining animals were grouped into 2 groups and each group was further sub-divided into 7 sub-groups. Among them, one sub-group was continued on LG diet and another sub-group was fed with AIN -76 basal diet and the other 5 sub-groups were fed with basal diet containing spices and SO as described earlier (Chapter IV) and indicated in the Fig. 5.1. One group of animals fed with experimental diets for 5 weeks (short-term) and another group for 10 weeks (long-term). Control animals were fed only AIN-76 basal diet throughout the duration of experiment.

Results

Confirmation of the formation of CGS in mice

At the end of the 10 weeks feeding of LG diet, 10 animals were randomly selected and sacrificed to confirm the formation of CGS. The LG diet feeding for 10 weeks successfully induced the formation of CGS in mice. The increase in the serum, liver and biliary cholesterol levels in mice preceded the CGS formation and is evident as shown in the Fig. 5.2. The CSI (also termed as the lithogenic index) of bile was >2 in the mice fed LG diet for 10 weeks. Thus, these results confirmed the formation of CGS by feeding LG diet confirming the results observed earlier (chapter-IV).

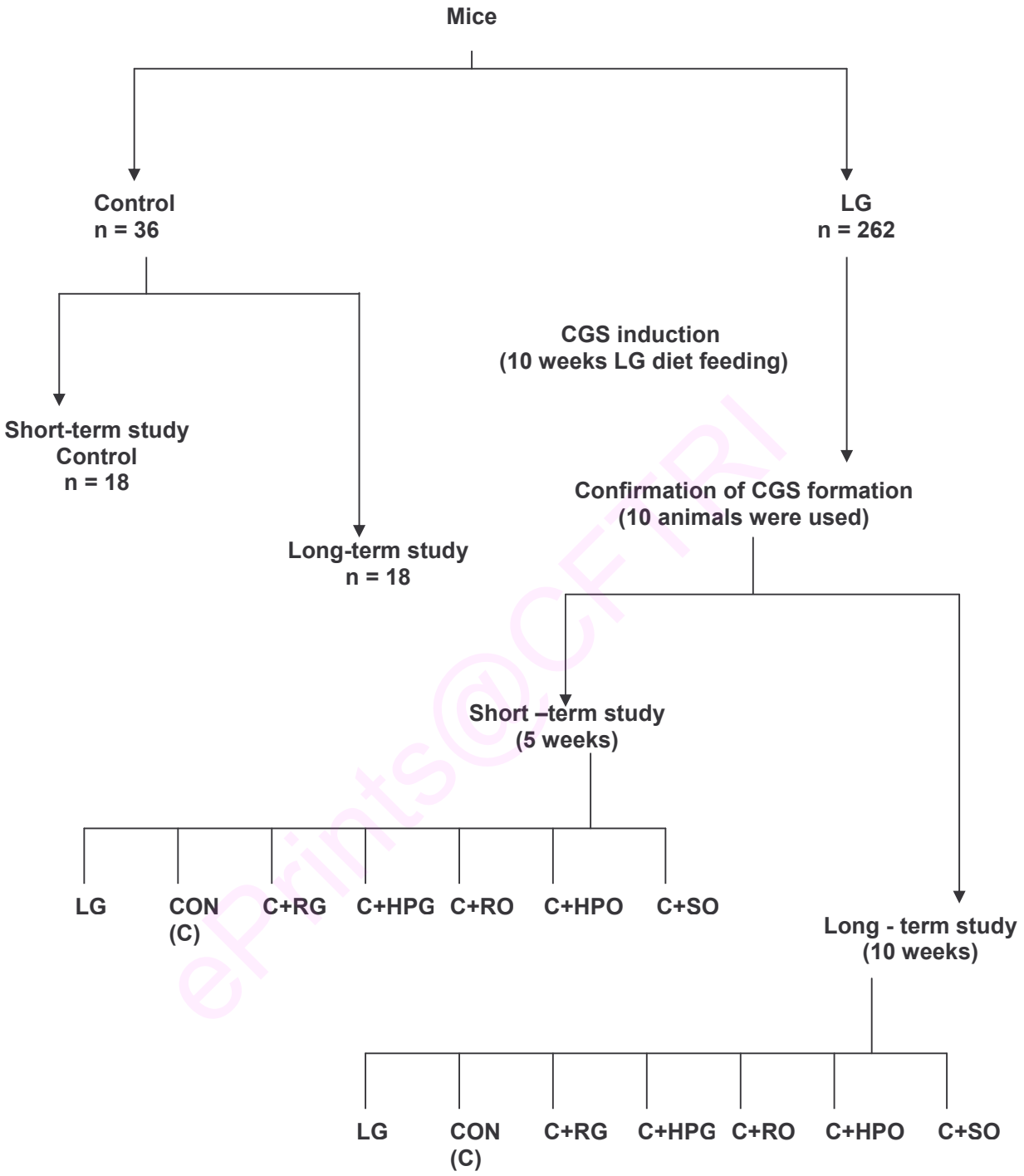


Fig 5.1: Experimental design for studying regression of CGS in mice by dietary spices and SO

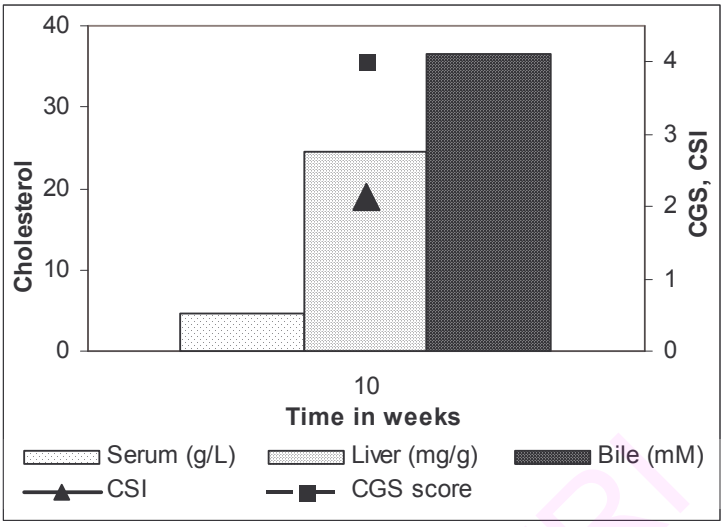


Fig 5.2: Relationship between serum, liver, biliary cholesterol and CGS Score and CSI of bile after 10 weeks feeding LG diet in mice

Short term studies (5 weeks)

Effect of short – term (5 weeks) feeding of spices and SO on growth parameters in CGS prevailing mice

After confirming the formation of CGS, the animals were grouped into groups and sub-groups as indicated in Fig. 5.1. One sub-group of animals was continued on LG diet and another sub-group was fed AIN-76 basal diet. The other five sub-groups were fed with spices (raw and heat processed garlic and onion) and sesame oil for 5 weeks and their effect on body, liver, gallbladder weight and CGS score was studied and results are given in Table-5.1. The body weights of the animals were comparable among the groups except for a slight increase in LG group. Liver weight was significantly increased in LG group by 2.2 times compared to control group. Whereas in C, RG, HPG, RO, HPO and SO groups decreased by 8, 22, 28, 22, 34 and 35% respectively compared to LG group. The gallbladder weight was significantly increased in LG group by 3 times and it was decreased by 8, 38, 31, 41, 50 and 35% in C , RG, HPG, RO, HPO and SO groups respectively compared to LG group. The CGS score was 3.80 in LG group and it was reduced to 3.65, 2.08, 3.25, 2.70, 2.0 and 2.75 in C, RG, HPG, RO, HPO and SO groups respectively. The CGS regression was 3.9, 45.3, 14.5, 28.9, 47.4 and 27.6 % in C, RG, HPG, RO, HPO and SO groups respectively (Fig-5.3).

Table 5.1: Effect of short – term (5 weeks) feeding of spices and SO on body weight, organ weights and CGS score in CGS prevailing mice

Dietary group	B.W (g)	Liver (g)	Gallbladder (mg)	CGS score	CGS occurrence (%)	CGS regression (%)
Control	43.0±6.23	1.43±0.12	23.92±4.61	-	-	-
LG	48.7±3.33	3.10±0.21[a]	71.17±4.15[a]	3.80±0.46[a]	100	-
C	45.6±2.36	2.84±0.31	65.45±2.13	3.65±0.56	96.0	3.9
C+RG	43.0±2.07	2.43±0.28[b]	43.95±5.90[b]	2.08±0.24[b]	54.7	45.2
C+HPG	43.7±3.56	2.24±0.58[b]	48.88±4.33[b]	3.25±0.41[b]	85.5	14.4
C+RO	42.1±2.17	2.40±0.47[b]	42.03±2.95[b]	2.70±0.21[b]	71.0	28.9
C+HPO	43.2±1.52	2.04±0.37[b]	35.71±3.71[b]	2.00±0.11[b]	52.6	47.3
C+SO	44.3±1.65	2.36±0.16[b]	40.23±2.30[b]	2.75±0.21[b]	72.3	27.6

Values are mean ± SD of 18 mice/group

C – Animals initially fed with LG diet to induce CGS formation and later discontinued LG diet and fed basal control diet only

Other group legends are as in Table-4.2

[a] Statistically significant when compared to control group at $P < 0.01$

[b] Statistically significant when compared to LG group at $P < 0.01$

CGS score – Values are average of 18 animals/group and scored by

4 different individuals grading was done on a 4 point scale (0-4)

Fig: 5.3: Effect of short - term (5 weeks) feeding of spices and SO on gallbladder size and gallstones during regression

Group legends are as in Table –5.1

Effect of short – term (5 weeks) feeding of spices and sesame oil on serum lipid profile in CGS prevailing mice

The spices and SO were fed for 5 weeks and their effect on serum lipids in CGS prevailing mice was studied and presented in Table-5.2. Cholesterol content of serum in mice of LG diet group was increased by 132 % compared to control group.

Table 5.2: Effect of short – term (5 weeks) feeding of spices and SO on serum lipid profile in CGS prevailing mice

Groups	Total cholesterol	Phospholipid	Triglycerides	C/PL
	(mg/dL)			
Control	195.4±9.23	323.6±19.34	157.9±16.89	0.60±0.04
LG	470.8±8.56 [a]	164.5±5.64[a]	195.6±12.23 [a]	2.86±0.31[a]
C	454.6±14.89	177.8±14.30	186.5±17.73	2.56±0.15[b]
C+RG	432.8±15.04[b]	274.3±12.07[b]	168.4±15.46[b]	1.58±0.13[b]
C+HPG	441.3±14.68[b]	205.2±17.09[b]	182.1±14.72[b]	2.19±0.31[b]
C+RO	431.0±11.88[b]	237.9±19.02[b]	172.8±6.88[b]	1.81±0.14[b]
C+HPO	415.4±13.30[b]	292.8±19.15[b]	177.1±14.67[b]	1.41±0.07[b]
C+SO	422.6±11.52[b]	289.5±15.02	172.4±11.21[b]	1.45±0.12[b]

Values are mean ± SD of 6 samples/group, each sample constituting 3 mice

[a] Statistically significant when compared to control group at $P < 0.01$

[b] Statistically significant when compared to LG group at $P < 0.01$

Group legends are as in Table –5.1

C/PL - cholesterol/phospholipid ratio

In mice fed spices and SO containing diets serum cholesterol was significantly decreased compared to LG group. The phospholipids content in RG, HPG, RO, HPO and SO groups was significantly increased by 54.3, 15.4, 33.8, 64.7 and 45% respectively compared to LG group. The C/PL was significantly increased in LG group due to increased cholesterol content and it was decreased in spices and SO groups significantly compared to LG group. These results indicated that spices and SO effectively decreased the serum triacylglycerol levels in mice compared to LG group. It is significant to note that control diet (C) feeding after CGS formation did not alter various lipid parameters in serum.

Effect of short – term (5 weeks) feeding of spices and sesame oil on liver lipid profile in CGS prevailing mice

Liver cholesterol content of LG diet fed mice was increased by 2.4 times compared to controls. The mice fed RG, HPG, RO, HPO and SO groups have decreased liver cholesterol content by 37, 18, 23, 36 and 15 % respectively compared to LG group (Table 5.3). Similarly triacylglycerol content was decreased by 22, 16, 20, 30 and 8% in RG, HPG, RO, HPO and SO groups respectively compared to LG diet group. The phospholipid content was not altered significantly among different groups. The C/PL ratio was significantly increased in LG group by 3.65 times indicating increased cholesterol content in liver compared to controls. In RG, HPG, RO, HPO and SO groups C/PL ratio was decreased by 38, 22, 27, 44 and 35% respectively compared to LG group. These results indicated that spices and SO could effectively decrease the liver cholesterol and triacylglycerol levels and also decreased the C/PL ratio in mice in CGS prevailing conditions. It is significant to note that control diet (C) feeding after CGS formation did not alter various lipid parameters in liver.

Table 5.3: Effect of short – term (5 weeks) feeding of spices and SO on liver lipid profile in CGS prevailing mice

Groups	Total cholesterol	Phospholipid	Triglycerides	C/PL
	(mg/g)			
Control	9.77±1.11	47.41±5.46	31.48±5.83	0.20 ± 0.03
LG	25.99±4.46[a]	36.15±4.62[a]	57.92±4.14[a]	0.73 ± 0.16[b]
C	24.12±3.56	36.15±2.35	54.12±3.21	0.66 ± 0.13
C+RG	17.92±2.94[b]	39.78±3.42	45.44±2.82[b]	0.45 ± 0.08[b]
C+HPG	21.22±1.81[b]	37.79±5.37	48.68±4.71[b]	0.57 ± 0.10[b]
C+RO	20.04±2.58[b]	37.37±3.80	46.33±3.72[b]	0.53 ± 0.06[b]
C+HPO	16.55±2.96[b]	40.41±3.82	40.58±4.78[b]	0.41 ± 0.08[b]
C+SO	19.85±3.21[b]	42.51±3.65	48.75±3.35[b]	0.46 ± 0.12[b]

Values are mean ± SD of 6 samples/group, each sample constituting 3 mice

[a] Statistically significant when compared to control group at $P < 0.01$

[b] Statistically significant when compared to LG group at $P < 0.01$

Group legends are as in Table –5.1

Effect of short – term (5-weeks) feeding of spices and SO on biliary lipid profile in CGS prevailing mice

Cholesterol content of bile was increased by 6.34 times in LG diet group compared to control group. On the other hand, biliary cholesterol content was decreased by 47, 32, 41, 56 and 15% in RG, HPG, RO, HPO and SO groups respectively (Table-5.4). RG, HPG, RO, HPO and SO feeding increased the bile acid content by 6, 4, 3, 14 and 8% respectively compared to LG group. The phospholipid content was not significantly altered among the groups. The C/PL ratio in LG group was increased significantly indicating increased cholesterol content. On the other hand C/PL ratio in spices and SO group was decreased by 22 - 44 % compared to LG group (Table-5.5). Similarly the C/BA ratio was increased to 0.19 in LG group and it was lowered in spices and SO fed groups (Table-5.5). The cholesterol saturation index (CSI) was 2.1 in LG group and it was decreased to 1.05, 1.26, 1.11, and 0.94 and 1.09 in RG, HPG, RO, HPO and SO fed groups respectively. The hydrophobicity index (HI) of bile was 0.053 in LG group and it was decreased to -0.07, -0.02, -0.05, -0.10 and -0.065 in RG, HPG, RO, HPO and SO groups respectively (Table-5.6). The content of hydrophilic bile acids TMC and TUDC were decreased in LG diet and they were increased by feeding spices and SO (Table-5.7). On the other hand, hydrophobic bile acids TC and TDC were increased in CGS prevailing mice and these were decreased by spices and SO significantly. The biliary phospholipid fatty acid profile showed that feeding spices and SO reduced the palmitic acid content by 17.6, 19.8, 20.7, 32 and 25% and increased the oleic acid content by 7.6, 5.1, 10.4, 16.0 and 13.0 % by RG, HPG, RO, HPO and SO fed groups respectively compared to LG group (Table-5.8). These results indicated that spices and SO could effectively decrease the cholesterol and increase the bile acids and phospholipids in gallbladder bile in CGS prevailing conditions. It is significant to note that control diet (C) feeding after CGS formation did not alter various lipid parameters.

Effect of short – term (5-weeks) feeding of spices and sesame oil on sterol metabolizing enzymes in CGS prevailing mice

Liver is an important organ involved in lipid and sterol metabolism. HMG-CoA reductase is the rate-limiting enzyme in the cholesterol biosynthesis. LG diet feeding significantly decreased HMG- CoA reductase activity. On the other hand feeding of spices and SO increased the activity by 3.6 to 5.1 times compared to LG diet. On the

Table 5.4: Effect of short – term (5-weeks) feeding of spices and SO on biliary lipid profile in CGS prevailing mice

Dietary group	Cholesterol	Phospholipid	Bile acids	Total lipid (g/dl)
	mM			
Control	5.25±0.25	12.74±1.52	200.7±22.25	11.04±1.12
LG	33.26±2.06[a]	22.17±2.29[a]	172.8±16.07[a]	11.49±0.85[a]
C	30.14±3.01	20.87±1.78	169.8±12.31	11.54±0.35
C+RG	17.57±1.77[b]	19.25±3.11	182.9±20.64[b]	11.15±1.15[b]
C+HPG	22.60±3.60[b]	23.09±2.72	180.1±11.02[b]	11.50±0.58[b]
C+RO	19.61±1.33[b]	21.60±2.57	178.5±20.19[b]	11.19±1.13[b]
C+HPO	14.75±0.93[b]	16.94±1.91[b]	196.7±19.48[b]	11.54±0.90[b]
C+SO	18.21±1.82[b]	20.18±2.61[b]	176.8±11.31[b]	11.46±0.48[b]

Values are mean ± SD of 6 samples/group, each sample constituting 3 mice

[a] Statistically significant when compared to control group at $P < 0.01$

[b] Statistically significant when compared to LG group at $P < 0.01$

Group legends are as in Table – 5.1

Table 5.5: Effect of short - term (5-weeks) feeding of spices and SO on biliary C/PL and C/BA ratio in bile in CGS prevailing mice

Dietary group	C/PL	C/BA
Control	0.41	0.03
LG	1.50	0.19
C	1.44	0.17
C+RG	0.91	0.10
C+HPG	0.98	0.13
C+RO	0.91	0.11
C+HPO	0.87	0.07
C+SO	0.90	0.10

Values are mean of 6 samples/group, each sample constituting 3 mice

C/PL - cholesterol/phospholipid

C/BA - cholesterol/bile acids

Group legends are as in Table – 5.1

Table 5.6: Effect of short – term (5-weeks) feeding of spices and SO on CSI and HI of bile in CGS prevailing mice

Dietary group	CSI	HI
Control	0.52±0.10	-0.164±0.033
LG	2.10±0.23[a]	0.0531±0.005[a]
C	1.95±0.33	0.045±0.05
C+RG	1.05±0.07[b]	-0.077±0.004[b]
C+HPG	1.26±0.10[b]	-0.026±0.007[b]
C+RO	1.11±0.13[b]	-0.057±0.005[b]
C+HPO	0.94±0.13[b]	-0.103±0.031[b]
C+SO	1.09±0.10[b]	-0.065±0.02[b]

Values are mean ± SD of 6 samples/group, each sample constituting 3 mice

[a] Statistically significant when compared to control group at $P < 0.01$

[b] Statistically significant when compared to LG group at $P < 0.01$

Group legends are as in Table – 5.1

CSI-Cholesterol saturation index

HI-Hydrophobic index

other hand enzymes involved in biosynthesis of bile acids from cholesterol, the cholesterol 7α-hydroxylase and sterol 27-hydroxylase were decreased during LG diet feeding. The RG, HPG, RO, HPO and SO feeding increased the activity of cholesterol 7α- hydroxylase by 62, 18, 39, 85 and 45 % respectively compared to LG group. Similarly RG, HPG, RO, HPO and SO increased sterol 27-hydroxylase activity by 22,15, 43, 55 and 35% respectively (Table- 5.9). It is significant to note that control diet (C) feeding after CGS formation significantly altered HMG-CoA reductase and sterol 27-hydroxylase activity.

Table 5.9: Effect of short – term (5-weeks) feeding of spices and SO on sterol metabolizing enzymes in CGS prevailing mice

Dietary groups	HMG-CoA Reductase*	Cholesterol 7α-hydroxylase**	Sterol 27-hydroxylase**
Control	57.63±5.62	28.53±1.62	63.48±2.68
LG	14.56±2.06[a]	9.63±1.38[a]	26.84±1.39[a]
C	19.85±1.65[b]	11.56±1.35	31.24±2.14[b]
C+RG	69.28±2.81[b]	15.62±1.93[b]	32.68±2.59[b]
C+HPG	52.64±3.65[b]	11.35±2.06[b]	30.82±4.13[b]
C+RO	58.21.±1.82[b]	13.35±1.94[b]	38.39±1.76[b]
C+HPO	73.65± 5.40[b]	17.82±1.63[b]	41.63±0.65[b]
C+SO	62.89± 4.56[b]	14.58±1.23[b]	31.25±1.32[b]

Values are Mean ± SD of 3 samples

* nmoles of CoA formed/min/mg protein

** pmoles of 7α-hydroxycholesterol and 27-hydroxycholesterol formed/min/mg protein

[a] Statistically significant when compared to control group at $P < 0.01$

[b] Statistically significant when compared to LG group at $P < 0.01$

Group legends are as in Table –5.1

Long-term studies (10 weeks)

Effect of long – term (10 weeks) feeding of spices and sesame oil on body and other organ weights in CGS prevailing mice

The effect of feeding spices (raw and heat processed garlic and onion) and sesame oil for 10 weeks on body, liver and gallbladder weight in CGS prevailing mice is given in Table- 5.10. The body weights of the animals were comparable among all the groups. Liver weight was significantly increased in LG diet group and feeding RG, HPG, RO, HPO and SO diet decreased the liver weight by 40, 41, 39, 45, 45 and 39% respectively compared to LG group. The gallbladder weight was increased in LG group by 3 times and feeding RG, HPG, RO, HPO and SO decreased the weight by 49, 42, 49, 58 and 38 % respectively. The CGS score was 1.85, 2.56, 2.3, 1.63 and 1.93 in RG, HPG, RO, HPO and SO groups respectively compared to 3.95 in LG group. The CGS regression was 53, 35, 42, 59 and 48 % in RG, HPG, RO, HPO and SO groups respectively (Fig-5.4). It is significant to note that control diet (C) feeding after CGS formation did not alter any of the parameters studied.

Effect of long – term (10 weeks) feeding of spices and SO on serum lipid profile in CGS prevailing mice

Cholesterol content of serum in LG diet fed group was increased significantly by 2.6 times compared to control group. The cholesterol content in mice fed RG, HPG, RO, HPO and SO containing diets was decreased by 28, 11, 16, 28 and 15 % respectively compared to LG group (Table - 5.11). Similarly triacylglycerol content was increased in LG diet group and it was significantly decreased in experimental diets fed groups. The RG, HPG, RO, HPO and SO increased phospholipid content by 41, 26, 27, 49 and 45 % respectively compared to LG group. The C/PL ratio was decreased by 49, 30, 34, 52 and 43 in RG, HPG, RO, HPO and SO respectively compared to LG group. These results indicated that spices and SO effectively decreased the serum cholesterol and triacylglycerol levels and C/PL ratio in mice in CGS prevailing conditions. It is significant to note that control diet (C) feeding after CGS formation showed tendency to decrease various lipid parameters studied but it was not statistically significant.

Table 5.10: Effect of long - term (10 weeks) feeding of spices and SO on body weight, organ weights and CGS score in CGS prevailing mice

Dietary group	B.W (g)	Liver (g)	Gallbladder (mg)	CGS score	CGS occurrence (%)	CGS regression (%)
Control	44.52±6.23	1.32±0.15	26.42±3.56	-	-	-
LG	46.33±2.26	3.52±0.31[a]	78.63±5.84[a]	3.95±0.23[a]	100	-
C	44.62±2.36	2.64±0.31[b]	55.45±2.13[b]	3.55±0.56	89.87	10.13
C+RG	41.65±3.1[b]	2.11±0.18[b]	39.86±3.36[b]	1.85±0.21[b]	46.83	53.17
C+HPG	42.88±2.18	2.09±0.18[b]	45.63±2.63[b]	2.56±0.32[b]	64.81	35.19
C+RO	40.20±1.93[b]	2.15±0.22[b]	40.15±3.10[b]	2.30±0.16[b]	58.22	41.78
C+HPO	40.32±1.36[b]	1.92±0.20[b]	33.10±2.80[b]	1.63±0.10[b]	41.26	58.74
C+SO	41.23±2.10	1.96±0.31[b]	42.31±1.65[b]	1.93±0.21[b]	52.31	47.69

Values are mean ± SD of 18 mice/group

[a] Statistically significant when compared to control group at $P < 0.01$

[b] Statistically significant when compared to LG group at $P < 0.01$

Group legends are as in Table –5.1

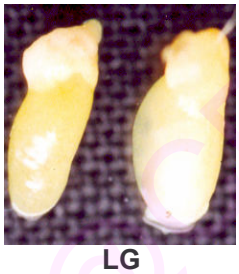
CGS score – Values are average of 18 animals/group scored by 4 different individuals and grading was done on a 0 - 4 point scale

Control

C

C+RG

C+HPG



LG

C+RO

C+HPO

C+SO

Fig 5.4: Effect of long-term (10 weeks) feeding of spices and SO on gallbladder size and gallstones during regression

Group legends are as in Table –5.1

Table 5.11: Effect of long - term (10 weeks) feeding spices and SO on serum lipid profile in CGS prevailing mice

Groups	Total cholesterol	Phospholipid	Triglycerides	C/PL
	(mg/dL)			
Control	181.9±14.95	316.4 ± 6.78	156.5 ± 8.04	0.57±0.04
LG	480.3±19.01[a]	200.3 ± 8.48[a]	174.3 ± 8.51[a]	2.40±0.18[a]
C	445.1±18.94	216.5±7.89	169.8±6.85	2.05±0.16
C+RG	347.2±28.80[b]	282.9 ±12.44[b]	154.8 ± 7.26[b]	1.23±0.13[b]
C+HPG	426.2±17.86[b]	252.7 ±19.82[b]	158.4 ± 7.80[b]	1.69±0.15[b]
C+RO	403.5±44.62[b]	254.0 ±11.81[b]	162.8 ± 6.10[b]	1.58±0.17[b]
C+HPO	345.7±35.09[b]	298.9±19.61[b]	160.1 ± 4.99[b]	1.15±0.06[b]
C+SO	378.9±23.35[b]	284.3 ±13.69[b]	165.4 ± 6.25[b]	1.33±0.21[b]

Values are mean ± SD of 6 samples/group, each sample constituting 3 mice

[a] Statistically significant when compared to control group at $P < 0.01$

[b] Statistically significant when compared to LG group at $P < 0.01$

Group legends are as in Table –5.1

C/PL - cholesterol/phospholipid ratio

Effect of long - term (10 weeks) feeding of spices and SO on liver lipid profile in CGS prevailing mice

The total cholesterol content in CGS containing mice was increased by 2.8 times compared to control diet fed mice. The cholesterol content of experimental diets fed mice decreased by 34, 23, 30, 38 and 25% in RG, HPG, RO, HPO and SO groups respectively compared to LG group (Table 5.12). Similarly increased triacylglycerol content was decresed by 15, 6, 10, 15 and 8% in RG, HPG, RO, HPO and SO groups respectively compared to LG group. RG, HPG, RO, HPO and SO increased the phospholipid content by 9, 10, 13, 25 and 12 % respectively compared to LG group. The RG, HPG, RO, HPO and SO decreased the C/PL ratio by 40, 29, 39, 51 and 35% respectively compared to LG group. These results indicated that spices and SO effectively decreased the liver cholesterol and triacylglycerol levels and C/PL ratio in mice with CGS prevailing conditions. It is significant to note that control diet (C) feeding after CGS formation showed a tendency to decrease various lipid parameters studied but it was not statistically significant.

Effect of long - term (10-weeks) feeding of spices and SO on biliary lipid profile in CGS prevailing mice

Cholesterol content of bile in CGS containing mice was higher by 5.7 times compared to control mice. The mice fed RG, HPG, RO, HPO and SO containing diets decreased cholesterol content in the bile by 53, 40, 47, 63 and 35 % respectively compared to LG group (Table-5.13). Marginal increase was observed in bile acids content in spices and SO groups compared to LG group. The phospholipid content varied little among various dietary groups. The RG, HPG, RO, HPO and SO decreased the C/PL ratio 38, 22, 27, 44 and 35 % respectively compared to LG group. Similarly the C/BA ratio was decreased by feeding spices and SO compared to LG group (Table-5.14). The cholesterol saturation index (CSI) was 2.14 in LG group and it was decreased to 0.97, 1.13, 1.04, and 0.91 and 0.99 in RG, HPG, RO, HPO and SO fed groups respectively. The hydrophobicity index of bile (HI) was 0.045 in LG group and it was decreased to -0.09, -0.03, -0.07, -0.12 and -0.08 in RG, HPG, RO, HPO and SO fed groups respectively (Table-5.15). The TMC and TMDC content in the bile was increased in spices and SO fed groups compared to LG group (Table-5.16). The biliary phospholipid fatty acid profile showed that feeding of RG, HPG, RO, HPO and SO reduced the palmitic acid content by 24, 16, 30, 33 and 25% and increased the oleic acid content by 9, 2, 11, 17 and 13% respectively compared

Table 5.12: Effect of long – term (10-weeks) feeding of spices and SO on liver lipid profile in CGS prevailing mice

Groups	Total cholesterol	Phospholipid	Triglycerides	C/PL
	(mg/g)			
Control	9.10±1.75	47.85±5.62	31.01±4.56	0.19±0.04
LG	25.74±2.70[a]	35.96±4.17[a]	52.38±4.75[a]	0.72±0.15[a]
C	21.10±2.26	34.12±2.55	49.87±3.21	0.61±0.13
C+RG	16.89±3.13[b]	39.26±3.48	44.66±3.10	0.43±0.09[b]
C+HPG	19.75±1.40[b]	39.46±5.22	49.42±5.15	0.51±0.10[b]
C+RO	17.88±2.39[b]	40.72±4.23	47.28±4.09	0.44±0.09[b]
C+HPO	16.04±2.57[b]	45.04±2.15[b]	44.32±6.72	0.35±0.06[b]
C+SO	18.59±1.65[b]	42.63±3.56[b]	42.65±3.65	0.43±0.12[b]

Values are mean ± SD of 6 samples/group, each sample constituting 3 mice

[a] Statistically significant when compared to control group at $P < 0.01$

[b] Statistically significant when compared to LG group at $P < 0.01$

Group legends are as in Table –5.1

Table 5.13: Effect of long - term (10-weeks) feeding of spices and SO on biliary lipid profile in CGS prevailing mice

Dietary group	Cholesterol	Phospholipid	Bile acids	Total lipid (g/dl)
	mM			
Control	5.54±0.46	13.83±1.33	200.4±10.53	11.12±0.54
LG	31.60±1.85[a]	24.11±1.62[a]	186.8±11.48[a]	12.26±0.62[a]
C	26.45±3.01	21.87±1.44	175.1±10.31	11.44±0.15
C+RG	14.88±1.54[b]	19.67±1.76	191.9±11.01	11.65±0.59
C+HPG	19.02±0.77[b]	22.68±2.07	198.0± 8.58	11.72±0.39
C+RO	16.88±1.01[b]	20.23±1.56[b]	193.3± 5.94	11.71±0.18
C+HPO	11.78±0.55[b]	18.04±1.95[b]	199.5±11.32	11.65±0.50
C+SO	15.86±1.19[b]	20.21±1.61[b]	194.3± 9.96	11.31±0.48

Values are mean ± SD of 6 samples/group, each sample constituting 3 mice

[a] Statistically significant when compared to control group at $P < 0.01$

[b] Statistically significant when compared to LG group at $P < 0.01$

Group legends are as in Table – 5.1

Table 5.14: Effect of long – term (10-weeks) feeding of spices and SO on biliary C/PL and C/BA ratio in CGS prevailing mice

Dietary group	C/PL	C/BA
Control	0.40	0.03
LG	1.31[a]	0.17[a]
C	1.20	0.15
C+RG	0.76[b]	0.08[b]
C+HPG	0.84[b]	0.10[b]
C+RO	0.83[b]	0.09[b]
C+HPO	0.65[b]	0.06[b]
C+SO	0.78[b]	0.08[b]

Values are mean of 6 samples/group, each sample constituting 3 mice

[a] Statistically significant when compared to control group at $P < 0.01$

[b] Statistically significant when compared to LG group at $P < 0.01$.

C/PL - cholesterol/phospholipid

C/BA - cholesterol/bile acids

Group legends are as in Table – 5.1

Table 5.15: Effect of long - term (10-weeks) feeding of spices and SO on CSI and HI of bile in CGS prevailing mice

Dietary group	CSI	HI
Control	0.53 ± 0.11	-0.15 ± 0.002
LG	2.14 ± 0.19[a]	0.045 ± 0.009[a]
C	1.84±0.12	0.024 ± 0.04
C+RG	0.97 ± 0.12[b]	-0.09 ± 0.003[b]
C+HPG	1.13 ± 0.06[b]	-0.03 ± 0.014[b]
C+RO	1.04 ± 0.13[b]	-0.07 ± 0.016[b]
C+HPO	0.91 ± 0.08[b]	-0.12 ± 0.038[b]
C+SO	0.99 ± 0.10[b]	-0.08 ± 0.003[b]

Values are mean ± SD of 6 samples/group, each sample constituting 3 mice

[a] Statistically significant when compared to control group at $P < 0.01$

[b] Statistically significant when compared to LG group at $P < 0.01$

Group legends are as in Table – 5.1

CSI-Cholesterol saturation index

HI-Hydrophobic index

to LG group (Table-5.17). It is significant to note that control diet (C) feeding after CGS formation showed tendency to decrease various lipid parameters studied but it was not statistically significant. These results indicated that spices and SO effectively decreased the cholesterol and increased the C/PL and C/BA ratios in gallbladder bile in CGS prevailing conditions.

Table 5.17: Effect of long – term (10-weeks) feeding spices and SO on biliary fatty acid composition (%) in CGS prevailing mice

Dietary group	16:0	16:1	18:0	18:1	18:2	20:4
Control	19.85±1.02	4.69±0.12	3.12±0.32	63.59±3.10	6.79±0.56	1.93±0.20
LG	30.28±2.31[a]	4.49±0.23[a]	3.59±0.54[a]	48.82±3.21[a]	9.85±0.45[a]	3.04±0.13[a]
C	28.56±1.51[b]	4.41±0.13	3.16±0.24[b]	52.36±2.51[b]	10.55±0.54[b]	2.41±0.32[b]
C+RG	22.98±2.05[b]	7.12±0.25[b]	5.91±0.45[b]	53.26±3.56[b]	8.56±0.98[b]	2.18±0.30[b]
C+HPG	25.56±1.36[b]	3.54±0.31[b]	1.54±0.21[b]	50.00±4.10[b]	11.62±1.54[b]	7.99±0.56[b]
C+RO	21.21±2.45[b]	3.56±0.13[b]	4.36±0.42[b]	54.04±2.30[b]	11.35±1.64[b]	5.48±0.41[b]
C+HPO	20.18±1.44[b]	3.05±0.21[b]	3.53±0.31	56.98±3.32[b]	9.54±0.87	6.72±0.32[b]
C +SO	23.56±2.31[b]	3.78±0.31[b]	3.69±0.54	56.95±3.21[b]	6.59±0.23[b]	5.64±0.16[b]

Values are Mean ± SD of 3 separate determinations.

[a] Statistically significant when compared to control group at $P < 0.01$

[b] Statistically significant when compared to LG group at $P < 0.01$

Group legends are as in Table –5.1

(10-weeks) feeding of spices and SO on sterol metabolizing enzymes in CGS prevailing mice

Liver is an important organ involved in lipid and sterol metabolism. HMG-CoA reductase is the rate-limiting enzyme in the cholesterol biosynthesis and the activity was significantly inhibited in LG diet fed group. The RG, HPG, RO, HPO and SO increased the HMG-CoA reductase activity in liver microsomes by 9, 7, 7, 10 and 4 folds respectively compared to LG group. On the other hand the cholesterol 7∝-hydroxylase and sterol 27-hydroxylase are important in the synthesis of bile acids from cholesterol. LG diet feeding significantly reduced activity of these enzymes. The RG, HPG, RO, HPO and SO feeding increased the activity of cholesterol 7∝-hydroxylase by 2.06, 1.56, 1.85, 2.52 and 1 fold respectively compared to LG group. RG, HPG, RO, HPO and SO feeding increased sterol 27-hydroxylase activity by 74, 45, 77, 118 and 64 % respectively compared to LG group (Table-5.18). It is significant to note that control diet (C) feeding after CGS formation significantly altered activities of all three sterol metabolizing enzymes. These results indicated that spices and SO effectively increased the activity of sterol metabolizing enzymes in CGS prevailing conditions in mice.

Relationship between serum, liver and bile cholesterol level and CSI and CGS score and effect of spices and SO

Cholesterol level in serum, liver and bile plays an important role in the formation of CGS. Relationship between serum, liver and bile cholesterol and CSI during regression of CGS by spices and SO feeding at two time intervals (short-term and long-term) is presented in Fig. 5.5, 5.6, and 5.7. These figures summarise the results presented in various tables and figures presented in this chapter. Cholesterol levels in serum, liver and bile were significantly higher in LG diet group compared to control group. Feeding spices and SO significantly reduced the cholesterol in serum, liver and bile, which is also reflected in the decreased CSI values compared to LG group at both time intervals. The ratio between C/PL and C/BA in bile plays an important role in determining the lithogenecity of bile. Hence relationship between C/PL and C/BA at two time intervals were given in Fig 5.8. The C/PL and C/BA ratio was decreased at both time intervals by feeding spices and SO. These results indicate the efficacy of spice and SO feeding at two time intervals in reducing the prevailing CGS. C diet feeding to CGS prevailing animals showed tendency to reduce various lipid parameters responsible for the formation of CGS.

Table 5.18: Effect of long – term (10-weeks) feeding of spices and SO on sterol metabolizing enzymes in CGS prevailing mice

Dietary groups	HMG-CoA reductase*	Cholesterol 7α-hydroxylase**	Sterol 27-hydroxylase**
Control	54.32±23.38	31.65±1.28	73.21±3.56
LG	9.29±1.97[a]	8.45±1.26[a]	22.80±1.39[a]
C	22.44±2.95[b]	14.19±2.33[b]	30.24±2.11[b]
C+RG	86.38±3.49[b]	17.45±2.36[b]	39.65±3.41[b]
C+HPG	64.83±2.83[b]	13.22±1.69[b]	33.16±2.62[b]
C+RO	67.29±2.31[b]	15.69±2.68[b]	40.36±0.98[b]
C+HPO	98.43±4.18[b]	21.36±2.69[b]	49.62±2.49[b]
C+SO	78.69±3.65[b]	16.89±3.64[b]	37.34±1.98[b]

Values are Mean ± SD of 3 samples

*nmoles of CoA formed/min/mg protein

**pmoles of 7α-hydroxychoelsterol and 27-hydroxycholesterol formed /min/mg protein

[a] Statistically significant when compared to control group at P < 0.01

[b] Statistically significant when compared to LG group at P < 0.01

Group legends are as in Table –5.1

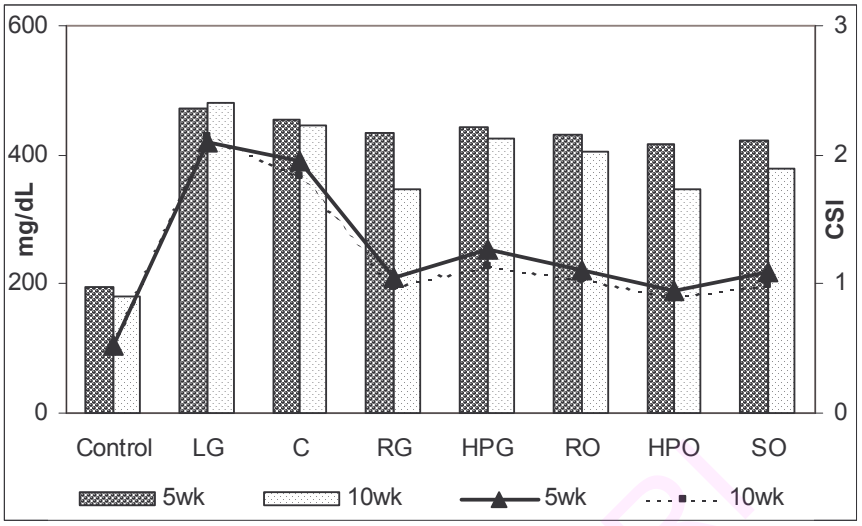


Fig 5.5: Relationship between serum cholesterol and CSI during regression of CGS in mice fed spices and SO

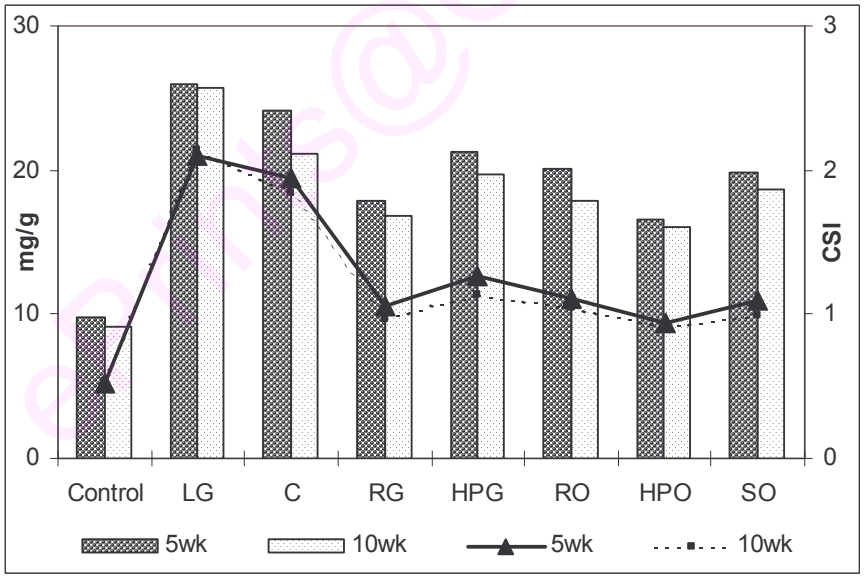


Fig 5.6: Relationship between liver cholesterol and CSI during regression of CGS in mice fed spices and SO

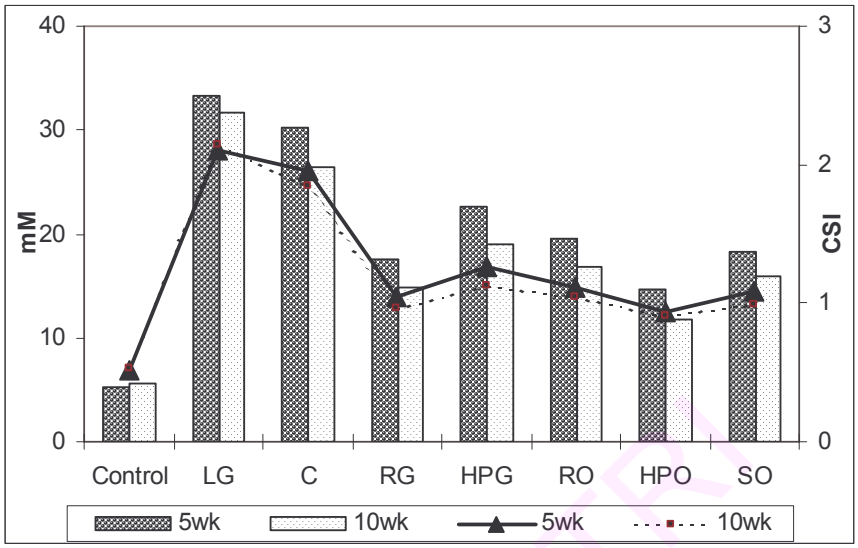


Fig 5.7: Relationship between biliary cholesterol and CSI during regression of CGS in mice fed spices and SO

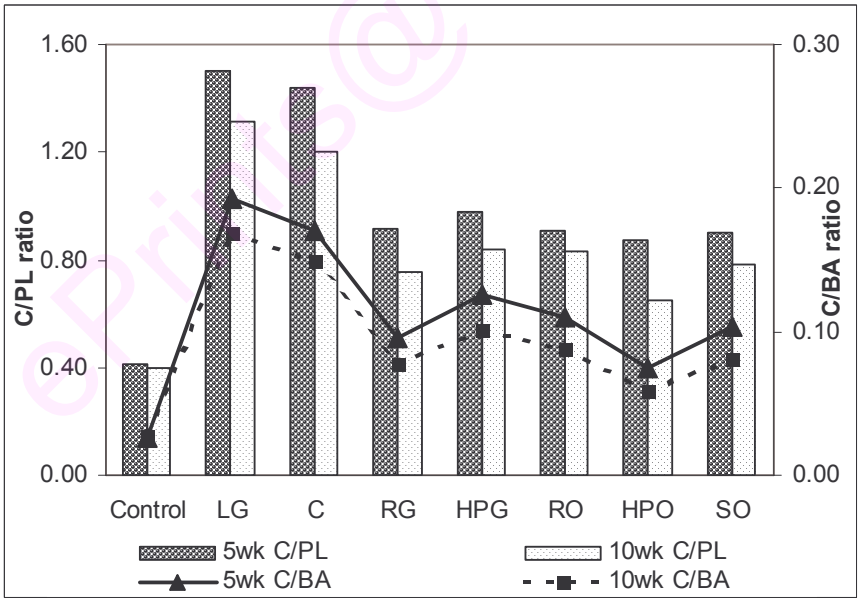


Fig 5.8: Relationship between bile C/PL and C/BA during regression of CGS in mice fed spices and SO

Effect of long-term (10 weeks) feeding of spices and SO on biliary lipid carriers in gallbladder bile in CGS prevailing mice

Cholesterol is carried in the form of micelles and vesicles in bile. Hence in the present investigation, the amount of cholesterol and phospholipid carried in the vesicles and micelles in mice bile was studied by separating vesicles and micelles by gel permeation chromatography in CGS prevailing conditions and the effect of long - term feeding (10 weeks) of spices and SO. The gallbladder bile when separated on gel filtration resolved into 3 peaks (Fig-5.9). The first peak eluting in the void volume was the vesicular peak, the other peak was micellar peak. The peak eluting between the vesicular and micellar peak was considered as inter-mixed micellar/vesicular or small vesicular (S-V) peak. The vesicular peak was significantly higher in LG group. Whereas, the S-V peak was absent in the control group bile. The relative areas of all the peaks in experimental groups were decreased compared to LG group.

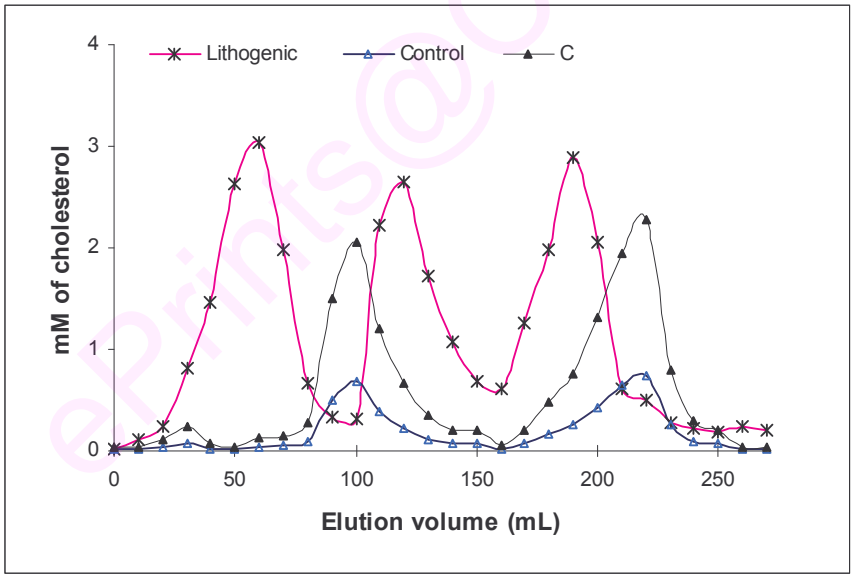


Fig 5.9: (A) Elution profile of mice gallbladder bile by gel permeation chromatography

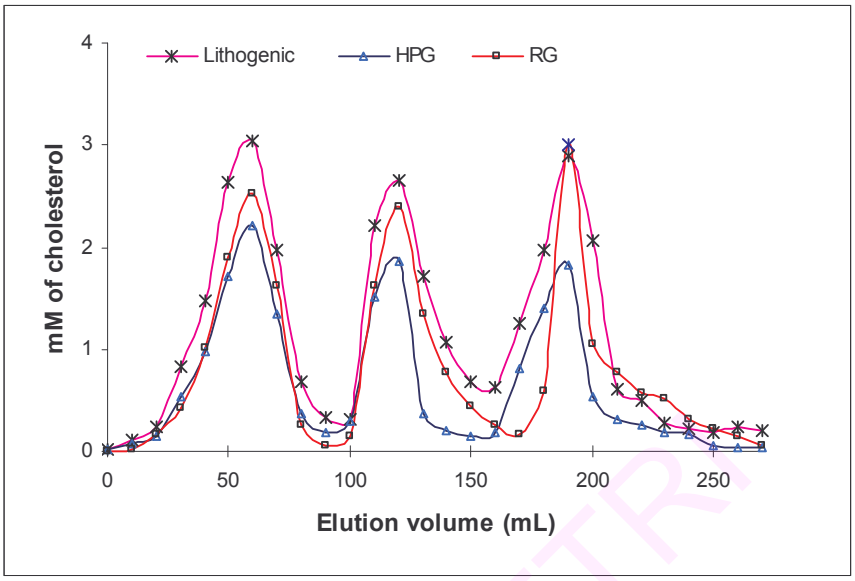


Fig 5.9: (B) Elution profile of mice gallbladder bile by gel permeation chromatography

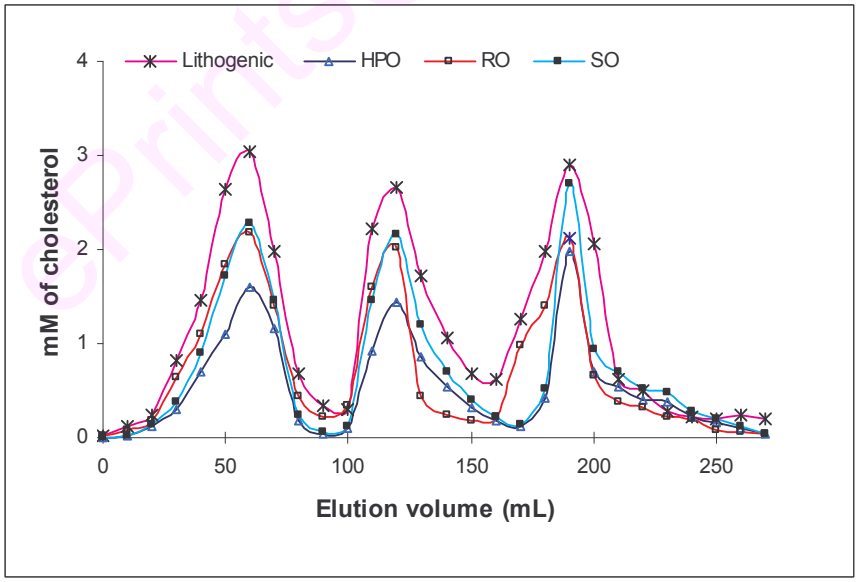


Fig 5.9: (C) Elution profile of mice gallbladder bile by gel permeation chromatography

The C/PL ratio in the vesicular fraction was highest (0.81) in LG group. It was reduced to 0.42, 0.45, 0.32, 0.41 and 0.49 by feeding RG, HPG, RO, HPO and SO diets respectively for 10 weeks in CGS induced mice. The C/PL ratio in the S-V fraction was highest (0.67) in LG group. It was reduced to 0.35, 0.60, 0.48, 0.44 and 0.48 by feeding RG, HPG, RO, HPO and SO diets respectively for 10 weeks in CGS induced mice. Similarly the C/PL ratio in the micellar fraction was 0.13 in LG group and It was 0.20, 0.10, 0.15, 0.15 and 0.14 by feeding RG, HPG, RO, HPO spice and SO for 10 weeks respectively in CGS induced mice. The amount of protein eluted in the micellar fraction was higher than vesicular fraction, which is followed by S-V fraction in all the dietary groups. The amount of cholesterol present in the vesicular fraction was higher than that of the S-V and micellar fraction respectively in all the dietary groups. Similarly, the amount of phospholipid present in the micellar fraction was higher than that of the vesicular and S-V fraction respectively in all the dietary groups (Table- 5.19). To summarize in spices and SO fed groups the biliary lipid contents of the micelllar and vesicular fractions were redistributed favoring solubilization of cholesterol in the bile.

Table 5.19: Effect of long - term (10-weeks) feeding of spices and SO on vesicular and micellar lipids in CGS prevailing mice bile

Dietary group	Cholesterol (mM)			Phospholipid (mM)			C/PL			Protein (mg/ml)		
	V	S-V	M	V	S-V	M	V	S-V	M	V	S-V	M
Control	-	0.86	2.31	4.2	3.1	5.40	-	0.28	0.43	-	0.96	0.89
LG	4.72	3.83	1.34	5.83	5.68	10.60	0.81	0.67	0.13	0.89	0.48	2.16
C	4.15	3.10	1.12	5.45	5.45	9.65	0.76	0.57	0.12	0.97	0.54	2.31
C+RG	2.48	1.49	1.86	5.84	4.28	9.21	0.42	0.35	0.20	1.12	0.78	2.24
C+HPG	3.71	2.68	1.08	8.16	4.44	10.40	0.45	0.60	0.10	1.46	1.23	3.11
C+RO	2.31	2.71	1.30	7.21	5.68	8.60	0.32	0.48	0.15	1.03	1.41	2.98
C+HPO	1.68	1.63	1.39	4.11	3.68	9.13	0.41	0.44	0.15	1.79	2.01	3.08
C+SO	1.75	1.43	1.24	3.54	2.98	8.98	0.49	0.48	0.14	1.35	1.98	2.64

Values are mean of 6 mice/group.

Group legends are as in Table –5.1.

V- Vesicles, S -V – Small vesicles, M - Micelles.

Discussion

Big and small CGS present in the gallbladder poses a major problem during their detection by the physicians as most of the CGS are radiolucent and cannot be detected easily. Their removal is usually carried out by either large doses of cholelitholytic drugs or by surgery [Roslyn et al, 1993]. Oral litholysis with ursodeoxycholic acid might still be appropriate for a subset of patients who do not want or are unfit for surgery, with small radiolucent cholesterol stones in a functioning gallbladder. Ursodeoxycholic acid could prevent gallstone formation in obese patients during rapid weight loss, but it is debatable whether ursodeoxycholic would also decrease the incidence of biliary symptoms in gallstone patients awaiting elective cholecystectomy. Gallstone recurrence after medical treatment together with cost-benefit analysis is the main reason why laparoscopic cholecystectomy has become the standard treatment of symptomatic gallbladder stones. But the major drawback of these two types of treatment is that the stones reappear upon discontinuation of the drug or after a lapse of time after lithotripsy [Bouchier, 1990].

A better method of treatment would be dietary intervention, which could help in prevention, regression of CGS and then stop recurrence. Data on the effects of dietary components on gallbladder bile in patients and in animals with established cholelithiasis are very limited. To study this hypothesis that dietary constituents can regress pre-formed CGS, studies were conducted by inducing CGS formation in gall bladder of mice with LG diet feeding and later looking for its regression under spices or SO feeding. As enumerated in the previous chapter, CGS formation was induced in 100 % of mice by feeding lithogenic diet for 10 weeks. After confirming the formation of CGS, LG diet was discontinued and they were fed control or experimental diets with or without raw and heat processed garlic, onion or sesame oil for 5 weeks (short duration) and 10 weeks (long duration). The results of the present investigation showed that on an average spices and SO feeding for 5 weeks after induction of CGS in mice was sufficient to cause regression of CGS up to 27% and long duration of feeding of spices and SO diet caused 47% regression.

Substitution of lithogenic diet with control diet to CGS mice neither caused any marked regression of CGS nor reduced the CSI and various lipid parameters in mice. From this it is clearly evident that mere withdrawal of lithogenic diet could not regress preformed CGS in the mice, whereas feeding spices or SO could influence both biliary lipid metabolism and CGS pathogenesis in particular.

Hussain and Chandrasekara (1994a,b) have reported that the curcumin and capsaicin regressed the pre-established CGS in mice significantly. Similarly fish oil feeding decreased the CSI by 25% after 5 weeks of treatment period in cholelithiasis prevailing patients [Berr et al, 1992]. On the other hand dietary supplementation of linoleic acid for 3 weeks did not alter the biliary lipid composition or CSI [Holan et al, 1979a].

Feeding of spices or SO resulted in a decrease in major part of the hepatic cholesterol pool, which is to be secreted into bile by its conversion to bile acids resulting in an increased bile acid concentration. Phospholipid concentration was also increased upon feeding spices and SO causing a phospholipid rich system. Increased bile acid and phospholipid concentration solubilize the biliary cholesterol in mixed micelles, which are stable and the possibility of nucleation of cholesterol crystals is reduced [Halpern et al, 1986b]. The cholesterol crystal inhibiting factors like proteins present in the bile was reported by many workers [Holzbach et al, 1973: Sedaghat and Grundy, 1980; Burnstein et al, 1985; Gallinger et al, 1985; Groen et al, 1988]. Our own observations with spices and SO here showed their influence in inhibiting cholesterol nucleation from the lipid carriers in the bile. These events affect the dynamic equilibrium of bile in such a way that the cholesterol supply to the growing CGS is cut-off and the CGS may degenerate or regress in the absence of continued supply of cholesterol and by the action of antinucleation proteins. Spices and SO reduce the total cholesterol pool, which is evident from the lower C: PL ratio of serum and liver.

Cholesterol is highly non-polar and is carried in the unsaturated bile (i.e. CSI<1) in the form of micelles containing phospholipid and bile salts. As the CSI rises above 1, bile is supersaturated with cholesterol. Then cholesterol is solubilized in unilamellar vesicles (i.e., bilayer structures composed of cholesterol and phospholipids, with small concentration of bile salts). Further fusion of unilamellar vesicles results in multilamellar vesicles (liquid crystals) resulting in the formation of solid cholesterol crystals. Hence the cholesterol transport in the micellar form is thermodynamically feasible for bile to be active physiologically [Portincasa et al, 2003]. In the present study an effort was made to understand whether spices and SO feeding have any influence on the lipid carriers and proteins present in bile and their role in CGS pathogenesis. Vesicles and small-vesicles with a high C/PL ratio were seen in the LG group. Vesicles with high C/PL ratio were known to nucleate to form cholesterol crystals [Lee et al, 1987; Harvey, 1987]. The micellar cholesterol shifts to an

intermediate structure, the S-vesicles to draw cholesterol from the micelles and later transfer them to the vesicles destined to nucleate cholesterol crystals. As the LG diet feeding was continued most of the cholesterol from the vesicles would have nucleated in the gallbladder and the cholesterol from the micelles is drawn towards vesicles. As a result, the micelles at this stage have a low cholesterol and phospholipid content. Finally the micelles disappear and most of the lipids appear in vesicles with high C/PL ratio as observed in the LG group. This confirms the earlier report that even micellar cholesterol nucleates via "the activation of vesicular pathway" [Peled et al, 1989]. In the group fed spice or SO, the vesicular peak contained lower amounts of cholesterol and phospholipid which indicates that a part of cholesterol and phospholipid have shifted from the unstable vesicles back to the stable micelles. Moreover, micelles transport 20 times more cholesterol than vesicles [Carey and Cohen, 1987]. There is a strong correlation between the % of regression and C/PL ratio of vesicles from which cholesterol precipitates directly. This reverse shift of cholesterol from vesicles to micelles can be studied by sequential chromatography of bile at different time intervals till the nucleation occurs in the incubated vial. This requires a large quantity of bile and mice model has a limitation for this type of work.

The difference in the lipid transport between the vesicles and micelles in the bile of different dietary groups cannot be explained on the basis of the protein eluting with vesicles and micelles, because irrespective of the dietary group, a large portion of the protein was eluted with micelles. One possible explanation is that the protein binding to the micelles in the LG group may destabilize the micelles by making a lipoprotein complex with either cholesterol or phospholipid [Dudley et al, 1986]. In the spice and SO fed group the protein binding to the micelles may stabilize it. Elution of the protein with either vesicles or micelles does not necessarily mean the protein is binding to the lipids. Whether a single protein or a group of proteins carry out the mechanism of lipid transfer between the vesicles and micelles *in vivo* is not known although some pure proteins like Con-A binding glycoproteins [Groen et al, 1988], Ig M [Harvey et al, 1991b], Apo A I and A II [Kibe et al, 1984] are known to carry out the mechanism of lipid transfer in model biles. It is possible that during purification several proteins not binding to the column of interest are lost which may be crucial in deciding the pronucleating and antinucleating effect in the cholesterol crystal nucleation process [Groen et al, 1988].

This study demonstrates that spices and SO have a profound effect on the transport of lipids from vesicles to micelles, which is probably facilitated by the biliary proteins. Considering the above facts it can be concluded that a consistent exchange and redistribution of biliary lipids between the carriers occur which is probably mediated by protein, thus inhibiting CGS formation or promoting the regression of CGS.

Chapter – VI

Studies on the effect of spices and sesame oil on biliary proteins and lipid peroxidation during CGS formation

Introduction

CGS occurs primarily due to supersaturation of cholesterol in bile leading to its precipitation (nucleation) in bile. Cholesterol precipitation is controlled by various factors such as mucin, glycoprotein, calcium ions, bilirubin and low molecular weight proteins present in the bile [Gallinger et al, 1986; Harvey et al, 1986]. Biliary proteins play a pivotal role in both promoting and inhibiting cholesterol precipitation. Biliary cholesterol supersaturation is frequently reported in normal as well as in patients with gallstones [Holzbach et al, 1973]. A rapid cholesterol nucleation time and an increased cholesterol crystal growth rate are two of the most important indices that influence gallstone formation and they are affected by cholesterol supersaturation [Holan et al, 1979b]. Apart from biliary lipid components, variety of biliary proteins can influence these two factors [Groen et al, 1988; Harvey and Strasberg, 1993; Harvey et al, 1991a; Holzbach et al, 1984]. Proteins that enhance the nucleation of cholesterol are called pronucleating and those which inhibit or prevent the nucleation of cholesterol are called antinucleating factors. Both pronucleation and antinucleation factors are found in human bile as well as in animals like mice, rats, gerbils and prairie dogs. In view of this concept, many proteins that bind to the lectin Con-A such as α-acid glycoprotein [Abei et al, 1993; 1994], hepatoglobin [Yamashita et al, 1995] and aminopeptidase-N 9 [Offiner et al, 1994] have been shown to promote cholesterol nucleation and crystal growth. Others binding to the Helix pomatia lectin (e.g., A 120 KD dimer) [Ohya et al, 1993] and a recently introduced family of cholesterol crystal associated proteins [Busch et al, 1994] appear to act as crystallization inhibitors. However with the sole exception of apolipoproteins, no inhibitor protein so far has been identified, fully characterized or quantified.

In contrast to the assumed direct interaction of glycoproteins during cholesterol crystal growth process, another biliary inhibitor protein, apo A-I has been reported either to partially shift the distribution of cholesterol in bile from vesicles towards the more stable mixed micellar form [Ahmed et al, 1994] or to stabilize cholesterol carriers such as phospholipids lamellae [Tao et al, 1993]. This increases the stability and appears to be based on its amphiphillic properties, allowing integration of apo A-I

into lipid aggregates. These studies indicate the complexity of the system in terms of the large number of factors which may affect cholesterol precipitation in bile.

Apart from these factors, yet another factor such as lipid peroxides in bile may play a crucial role in the cholesterol crystallization [Eder et al, 1995]. Since an increase in the fraction of peroxidized lipids in the aged and the increasing frequency of gallbladder stone formation in the elderly supports this hypothesis.

In the previous chapters it was shown that spices (garlic and onion) and SO were effective in reducing the formation of CGS and also in the regression of the pre-formed CGS. Hence, to understand the mechanism of cholesterol crystal nucleation and the effect of proteins isolated from bile of rats fed spices and SO, these were tested with supersaturated model biles for cholesterol nucleation. The role of lipid peroxidation on the onset of cholesterol nucleation in rat bile fed spices and SO was also studied in normal and induced conditions.

Experimental

Male albino rats (70) weighing about 140 g were used in the experiment. The rats were grouped into 7 groups with 10 animals/group and fed with different experimental diets (as described earlier) for 8 weeks. At the end of the experimental period the bile was collected after bile cannulation as described previously (chapter II).

Mixing experiment

Hepatic bile from LG diet group and various dietary groups were mixed in various ratios of 100:0, 90:10, 75:25, 50:50, 25:75, 10:90 and 0:100 and incubated with 0.04% sodium azide in sterile tubes with air tight caps for 21 days at 37°C. At different time intervals an aliquot of bile was taken and observed under polarized microscope. The time at which the appearance of any crystal observed was considered as the nucleation time of that particular bile sample. At the end of the incubation period all the bile samples were extracted and lipid composition was determined. CSI was also calculated from the lipid composition for pure as well as mixed bile samples.

Biliary lipid peroxidation induced by H_2O_2 in rat bile

The hepatic bile was incubated in two sets with H_2O_2 (0.1mM), $FeSO_4$ (0.2mM) and ascorbic acid (0.25mM) for 30 min for the induction of lipid peroxidation. One set was used for total lipid extraction and used for measuring lipid peroxidation. The other set was used to study the nucleation of cholesterol at different time intervals by incubating at 37°C.

Crystal growth assay

The crystal growth assay was carried out in two sets viz, seeded and unseeded to study the effect of different biliary protein fractions from different dietary groups on nucleation of cholesterol crystal and other parameters. The seeded set is one to which 10 μg of cholesterol crystal /mL bile was added whereas the unseeded set model bile alone was used.

Nucleation of vesicles and micelles

The model bile was prepared as described in chapter II and subjected to gel filtration chromatography to separate vesicles and micelles as described earlier. The obtained vesicles and micelles were incubated with Con-A bound LMW biliary protein (200 μg)

separated from different dietary groups to study the effect of spices and SO on nucleation of vesicles and micelles.

Molecular weight determination by electrophoresis

The approximate molecular weight of Con-A bound protein fraction was determined by SDS-gel electrophoresis as described.

Results

Effect of spices and SO on nucleation time in different bile mixtures

Hepatic bile of rats from various dietary groups as such and intermixed were incubated and the time at which the appearance of any crystal observed was considered as the nucleation time of that particular bile sample. The rat bile composition, CSI and nucleation time is given in Table-6.1. Results showed that lithogenic diet group bile with CSI of 2.16 was nucleated on 5th day. On the other hand, nucleation of cholesterol in bile of rats fed with RG, HPG, RO, HPO and SO occured on 15, 11, 14, 18 and 15th day respectively. Their CSI was also significantly lower compared to LG diet group. Similarly the nucleation time of the mixture containing lithogenic group bile and spices and SO groups bile in different ratios is given in Table-6.2. The nucleation time was increased as the content of the spices and SO group bile content increased in the mixed bile.

Effect of spices and SO on lipid peroxidation and cholesterol nucleation time

The lipid peroxidation may enhance the cholesterol nucleation time in bile leading to the CGS pathogenesis. Hence the hepatic bile from different dietary groups was analysed for the extent of lipid peroxidation and cholesterol nucleation time in normal and induced conditions (Table-6.3). The lipid peroxides were 80 and 57% higher in the LG group in normal and induced conditions respectively compared to control group. The lipid peroxidation was lowered by 28, 24, 26, 33 and 29 % in RG, HPG, RO, HPO and SO groups respectively compared to LG group under normal conditions. Similarly, the level of lipid peroxides were lowered by 20, 12, 16, 26 and 17 % in RG, HPG, RO, HPO and SO group respectively compared to LG group under induced conditions. The nucleation of cholesterol in normal bile was observed at 18 days compared to 7 days in LG group. Whereas, nucleation of cholesterol was observed on 12, 8, 10, 14 and 11 days in RG, HPG, RO, HPO and SO group respectively (Table-6.4). Similarly, under induced conditions the nucleation of cholesterol in normal bile was observed at 14 days compared to 5 days in LG group. Whereas, nucleation of cholesterol under induced conditions was observed on 9, 7, 8, 11 and 9 days in RG, HPG, RO, HPO and SO groups respectively. These results indicated that nucleation times were lowered in bile samples where the lipid peroxides are more. Feeding of spices and SO decreased the lipid peroxides in bile both in normal and induced conditions and enhanced the nucleation time of cholesterol in bile.

Table 6.2: Nucleation of cholesterol crystals in the bile of rats fed different diets

Dietary group	Nucleation time (days)							Dietary group
	100:0	90:10	75:25	50:50	25:75	10:90	0:100	
LG	6	6	8	14	16	ND	ND	Control
"	5	7	11	15	18	ND	ND	RG
"	6	6	9	14	16	20	21	HPG
"	6	7	10	15	17	20	-	RO
"	6	8	11	16	19	-	-	HPO
"	6	6	9	15	17	21	-	SO

ND – Crystals not detected even on 21 days

Each value is the mean of 3 samples

Group legends are as in Table – 5.1

Table 6.3: Effect of spices and SO on lipid peroxidation induced by H_2O_2 in rat bile

Groups	Uninduced	Induced (with H_2O_2)
MDA (n moles/dL)		
Control	110.36 ± 3.89	168.8 ± 3.45
LG	198.8 ± 5.77[a]	265.3 ± 7.89[a]
RG	143.3 ± 8.34[b]	210.8 ± 8.78[b]
HPG	151.6 ± 9.44[b]	234.3 ± 6.44[b]
RO	147.3 ± 6.21[b]	221.6 ± 11.42[b]
HPO	132.5 ± 3.29[b]	196.7 ± 7.47[b]
SO	140.8 ± 8.34[b]	216.9 ± 6.76[b]

Values are mean ± SD of 6 samples/group.

[a] Statistically significant when compared to control group at $P < 0.01$

[b] Statistically significant when compared to LG group at $P < 0.01$

Group legends are as in Table – 5.1

Table 6.4: Effect of lipid peroxides induced by H_2O_2 on bile of rats fed spices and SO on cholesterol nucleation time

Groups	Uninduced	Induced (with H_2O_2)
Nucleation time in days		
Control	18	14
LG	7	5
RG	12	9
HPG	8	7
RO	10	8
HPO	14	11
SO	11	9

Values are mean ± SD of 6 samples/group.

Group legends are as in Table – 5.1

Relationship between biliary lipid peroxidation and cholesterol nucleation time

The extent of lipid peroxidation is inversely related to the cholesterol nucleation time. LG group bile is more prone to peroxidation in both normal and induced conditions compared to control and the cholesterol nucleation time is decreased as shown in Fig 6.1. The extent of lipid peroxidation under normal and induced condition was lower in RG, HPG, RO, HPO and SO groups compared to LG group as shown in Fig 6.1. Hence it is evident that spices and SO effectively reduced the biliary lipid peroxidation and increased the cholesterol nucleation time in rat hepatic bile.

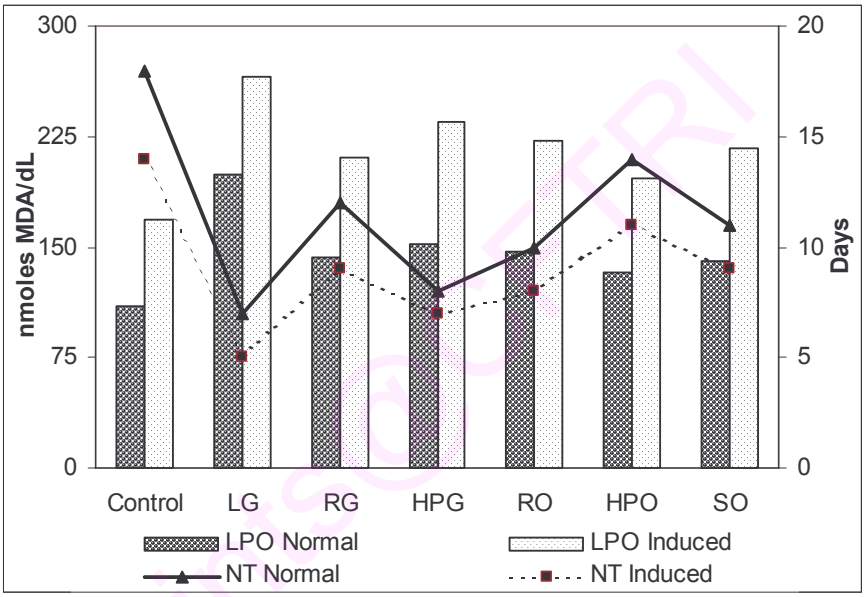


Fig 6.1: Relationship between biliary lipid peroxidation and cholesterol nucleation time

NT- cholesterol nucleation time

Effect of biliary protein fractions from spices and SO fed groups on supersaturated model bile

Effect of HMW fraction –

The crystal growth assay was carried out in two sets, viz: unseeded and seeded. Results in Fig-6.2 shows that addition of HMW fraction obtained from the lithogenic group shortened the nucleation time, increased the growth rate and final crystal concentration of the unseeded model bile. The crystal observation time was 10 h in the lithogenic group HMW fraction. The effect of HMW fractions from the groups fed with lithogenic diet supplemented with spices and SO was opposite to that of lithogenic group HMW fraction. The nucleation time was prolonged and it was between 15-20 h in spices and SO fed groups as shown in Fig-6.2.

The seeded set is one to which 10 μg of cholesterol crystal/mL model bile was added. Addition of cholesterol crystals abolishes the event of nucleation. In the seeded set of model bile the addition of HMW protein from LG group increased the crystal growth rate and final crystal concentration. But LG diet group supplemented with spices and SO brought down the crystal growth rate and final crystal concentration (Fig-6.3).

The maximal crystal growth rate (I_g), final crystal concentration (I_c) and onset time of crystal detection (I_t) in the model bile was determined after addition of 250 μg of HMW fraction in the unseeded set. The I_g value was 1.54 in LG group and it was reduced to 1.31, 1.5, 1.42, 1.18 and 1.23 in RG, HPG, RO, HPO and SO groups respectively (Table-6.5). The I_c value was 1.64 in LG group and it was significantly reduced to 1.33, 1.57, 1.47, 1.15 and 1.26 in RG, HPG, RO, HPO and SO fed groups respectively (Table-6.5). The I_t value was 1.0 in LG group and it was significantly increased to 2.5, 1.5, 2.0, 2.5 and 2.0 in RG, HPG, RO, HPO and SO fed groups respectively (Table-6.5). On the other hand, in the seeded set I_g and I_c values were not altered significantly by the addition of HMW fraction. Hence feeding spices and SO markedly reduced the I_t, I_g and I_c values in unseeded sets.

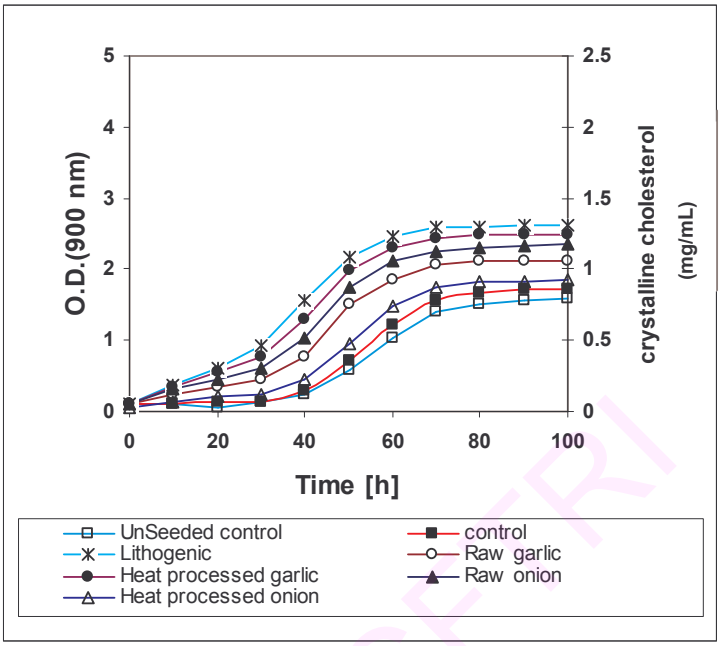


Fig 6.2: Effect of HMW biliary protein on crystal growth in unseeded set

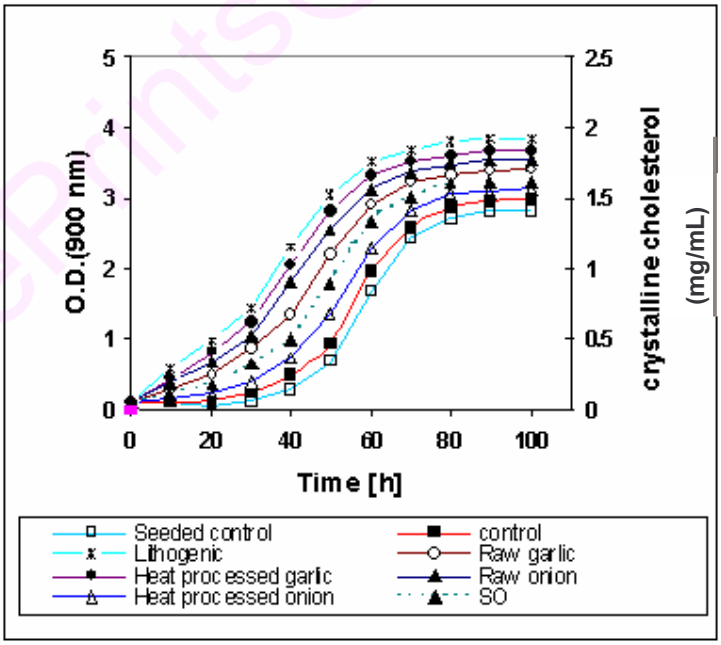


Fig 6.3: Effect of HMW biliary protein on crystal growth in seeded set

Table 6.5: Influence of HMW protein fractions from bile of rats fed different diets on crystal growth parameters tested in model bile

Dietary groups	Protein Conc. (µg)	Seeded		Unseeded		
		I_g	I_c	I_g	I_c	I_t
Control	250	1.05	1.04	1.08	1.08	2.5
LG	250	1.23	1.35	1.54	1.64	1.0
RG	250	1.15	1.20	1.31	1.33	2.5
HPG	250	1.19	1.29	1.50	1.57	1.5
RO	250	1.17	1.25	1.42	1.47	2.0
HPO	250	1.09	1.09	1.18	1.15	2.5
SO	250	1.12	1.18	1.23	1.26	2.0

Each value is the mean of 3 samples.

I_g-Growth Index, I_c- Crystal Index, I_t- Onset time of crystal detection

Data calculated from Fig 6.2 & 6.3

Group legends are as in Table – 5.1

Effect of LMW fraction –

Results in Fig-6.4 shows that addition of LMW fraction obtained from the lithogenic group shortened the nucleation time, increased the crystal growth rate and final crystal concentration of the unseeded model bile. The crystal observation time was 1 h in the lithogenic group. The effect of LMW fractions from the groups fed with lithogenic diet supplemented with spices and SO was opposite to that of lithogenic group fraction. The nucleation time was prolonged in spices and SO fed groups in unseeded set.

In the seeded set of model bile the addition of LMW protein from LG group increased the crystal growth rate and final crystal concentration. But LG diet group supplemented with spice and SO brought down the crystal growth rate and final crystal concentration Fig-6.5.

In the unseeded set the I_g values of RG, HPG, RO, HPO and SO were 1.27, 1.33, 1.31, 1.20 and 1.31 respectively compared to 1.36 in LG group (Table-6.6). The I_c values of the RG, HPG, RO, HPO and SO were 1.36, 1.50, 1.44, 1.22 and 1.41 respectively compared to 1.58 in LG group in the unseeded set. The I_t values for the RG, HPG, RO, HPO and SO were 3.0, 2.0, 2.5, 3.4 and 3.0 respectively compared to 1.0 in LG group in the unseeded set. On the other hand, in the seeded set the I_g and I_c values were not altered by the addition of LMW fraction. Thus the effect of feeding spices and SO was markedly pronounced in the reduced I_t values in unseeded set.

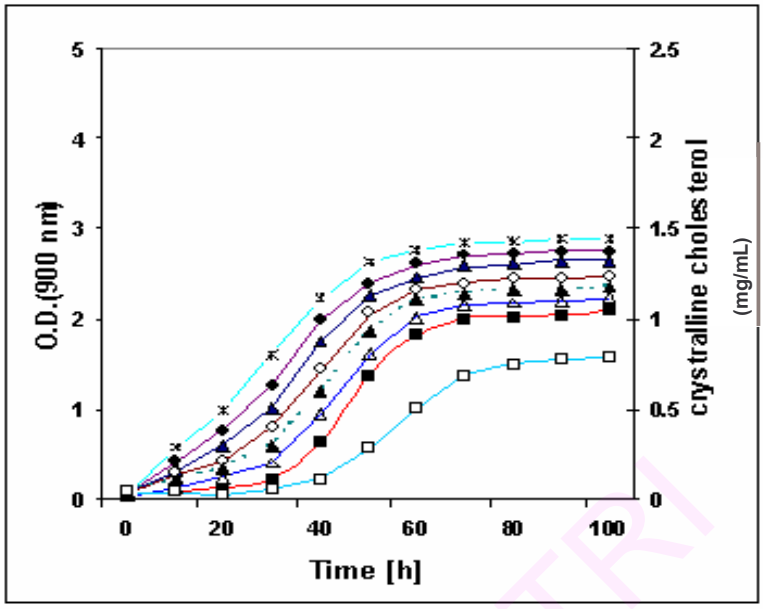


Fig 6.4: Effect of LMW biliary protein on crystal growth in unseeded set

Legends are as in Fig-6.2

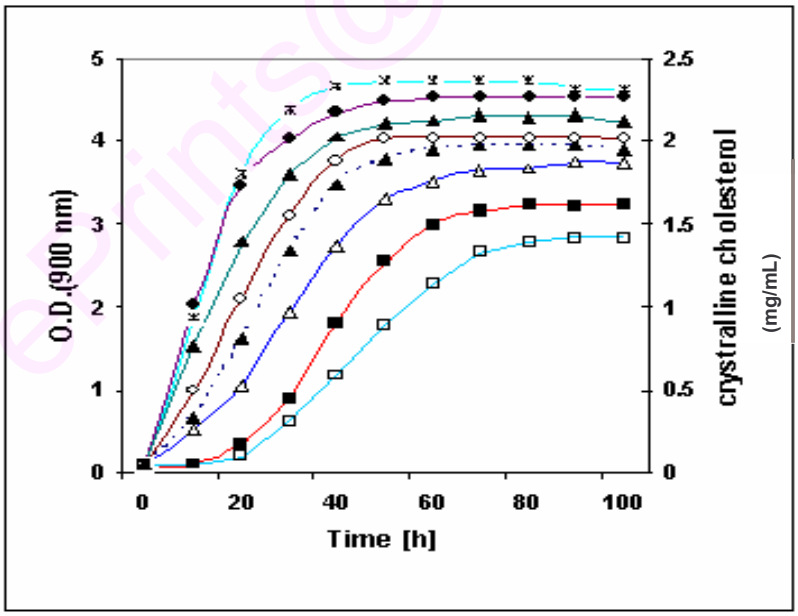


Fig 6.5: Effect of LMW biliary protein on crystal growth in seeded set

Legends are as in Fig-6.3

Table 6.6: Influence of LMW protein fractions from bile of rats fed different diets on crystal growth parameters tested in model bile

Dietary groups	Protein Conc. (µg)	Seeded		Unseeded		
		I_g	I_c	I_g	I_c	I_t
Control	250	1.16	1.15	1.13	1.14	2.0
LG	250	1.19	1.66	1.36	1.58	1.0
RG	250	1.17	1.44	1.27	1.36	3.0
HPG	250	1.16	1.62	1.33	1.50	2.0
RO	250	1.15	1.51	1.31	1.44	2.5
HPO	250	1.21	1.33	1.20	1.22	3.4
SO	250	1.14	1.30	1.31	1.41	3.0

Each value is the mean of 3 samples

Data calculated from Fig-6.4 & 6.5

Group legends are as in Fable – 5.1

Effect of glycoprotein fractions separated by affinity lectin chromatography

The LMW protein fractions obtained from different dietary groups on gel filtration were further purified on Con-A lectin affinity column to separate sugar specific glycoproteins (Fig-6.6). The concentration of the protein bound to Con-A column was higher in lithogenic group compared to other spices and SO fed groups. Addition of Con-A bound fraction from lithogenic diet fed group bile to unseeded supersaturated model bile shortened the nucleation time, increased the crystal growth rate and final crystal concentration (Fig-6.7). When 100 μg of Con-A bound fraction from spices and SO groups was added to model bile, the nucleation time was 10-15 h and the crystal growth rate and final crystal concentration was lesser than that of the lithogenic group. On the other hand addition of Con-A bound fraction from lithogenic group to the seeded set has the higher crystal growth rate and final crystal concentration (Fig-6.8). But addition of Con-A bound fraction from spices and SO group reduced the crystal growth rate and final crystal concentration. These results showed that biliary proteins from lithogenic group accelerated the nucleation of cholesterol crystal and crystal growth whereas the proteins isolated from spice and SO group inhibited the nucleation and crystal growth rates.

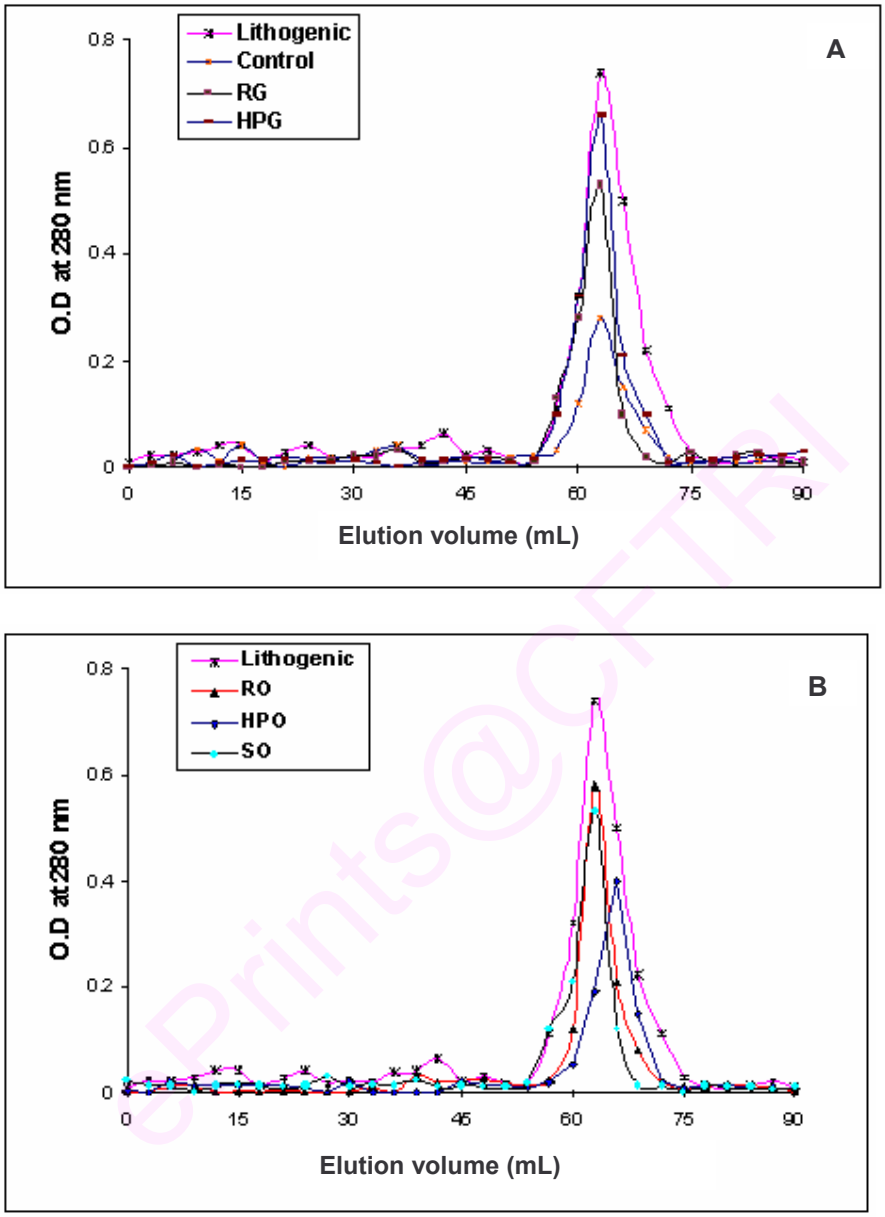


Fig 6.6 (A&B): Affinity lectin column (Con - A) chromatography of LMW protein fractions of bile obtained from different dietary groups

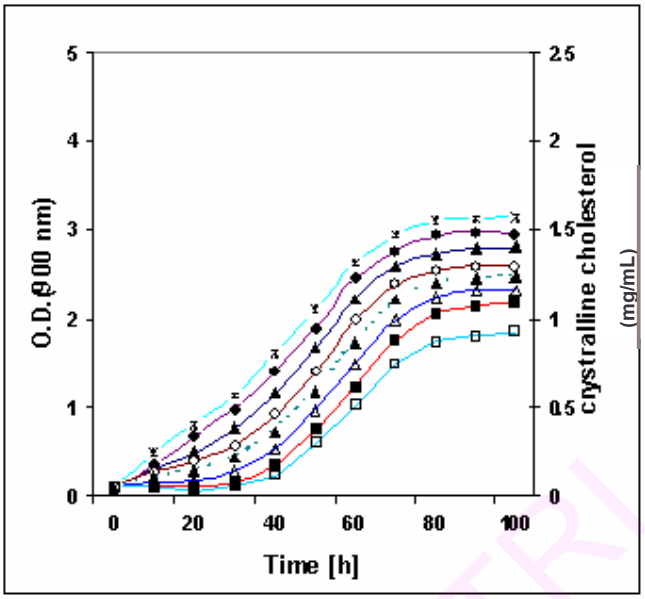


Fig 6.7: Effect of Con-A bound biliary protein fraction on crystal growth in unseeded set

Legends are as in Fig 6.2

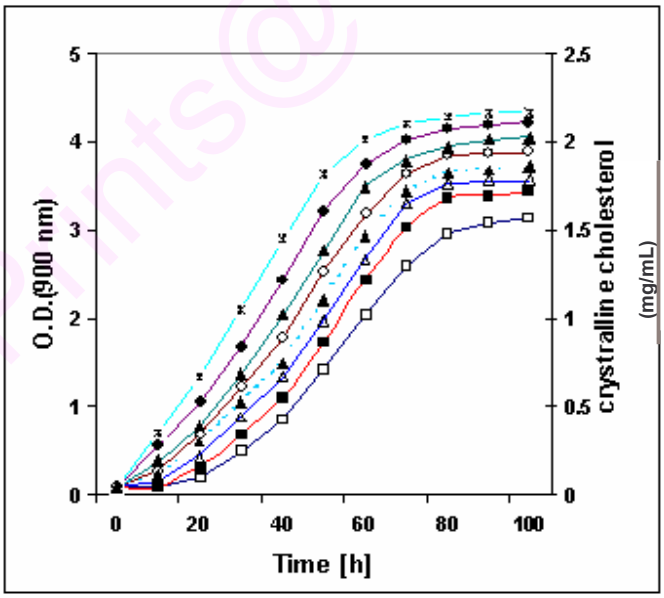


Fig 6.8: Effect of Con-A bound biliary protein fraction on crystal growth in seeded set

Legends are as in Fig 6.3

Effect of Con-A bound glycoprotein fraction on nucleation time of vesicles and micelles

Cholesterol is highly insoluble in aqueous medium of the bile and it is transported in the form of cholesterol-phospholipid structures called vesicles and cholesterol-phospholipid-bile salt structures called micelles. Cholesterol crystal nucleation occurs from vesicles. In this context, the effect of Con-A bound glycoprotein fraction isolated from rat bile fed spices and SO on cholesterol crystal nucleation was studied in vesicles and micelles separated from model bile by gel filtration. Initially no crystals were seen in either vesicles or micelles. Vesicles and micelles separated from the model bile nucleated on 12^{th} and 18^{th} day respectively (Table – 6.7). When 200 μg Con-A bound fraction isolated from lithogenic group was added to vesicles or micelles cholesterol nucleation occurred on 5^{th} day and 10^{th} day respectively. Similarly when spices and SO group protein fraction was added to vesicles, the cholesterol nucleation time was prolonged and it was nucleated on 9, 7, 8, 12 and 9^{th} day in RG, HPG, RO, HPO and SO group respectively. The micellar fraction nucleated on 19, 15, 16, 20 and 18^{th} day in RG, HPG, RO, HPO and SO group respectively (Table-6.7).

Table 6.7: Effect of Con-A bound protein fraction from the bile of rats fed various diet on the nucleation time on vesicle and micelles separated from model bile

Dietary groups	Nucleation time (days)		Protein (µg)
	Vesicles	Micelles	
Model Bile	12	18	-
Control	16	ND	200
LG	5	10	200
RG	9	19	200
HPG	7	15	200
RO	8	16	200
HPO	12	20	200
SO	9	18	200

Each value is the mean of 3 samples

Group legends are as in Table – 5.1

Electrophoresis of Con-A bound LMW fraction from spices and SO fed rat bile

The LMW fraction obtained from rat bile from different dietary groups resolved into several bands on electrophoresis Fig 6.9. There was a distinct difference between gel patterns between the groups. The Con - A bound LMW fraction resolved on the gel showed several bands and a band with a molecular weight 24 KD was more in the lithogenic group. The concentration of the same band in the spices and SO groups failed to express in the similar concentration. This indicated that this protein might be responsible for pronucleating activity when fed lithogenic diet. But feeding spice and SO inhibited the expression of this protein.

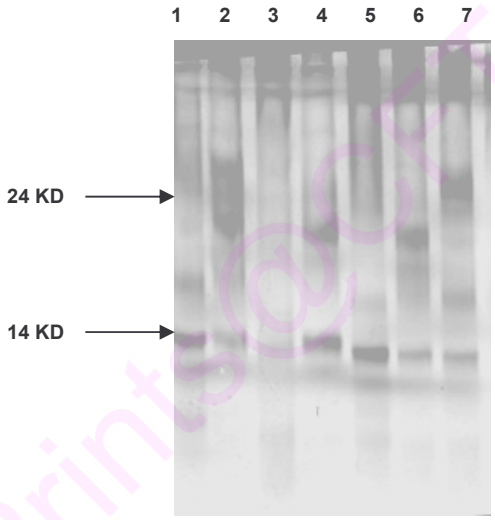


Fig 6.9: SDS-gel electrophoresis of Con A bound bile protein fraction separated from different spices and SO fed groups.
(1) Control (2) LG (3) RG (4) HPG (5) RO (6) HPO (7) SO

Group legends are as in Table – 5.1

Discussion

It has been reported earlier by several people that biliary proteins influence cholesterol crystal nucleation but the crystallization sequence due to proteins is not completely clear**.** Reports regarding pronucleating proteins such as mucin [Lee et al, 1989; Smith, 1990] and other glycoproteins [Burnstein et al, 1983; Lee et al 1989] thought to provide a nucleating matrix for cholesterol crystal formation. IgM and IgA can serve as pronucleating agents [Harvey et al, 1991b]. The antinucleation proteins such as apolipoproteins (Apo-AI, AII) are also found in bile. The bile samples from gallbladders of gallstone patients contain cholesterol crystals [Sedaghat and Grundy, 1980; Yamazki et al, 1988], which cannot be completely removed with the result that reliable crystal growth assays cannot be performed. Further procedures used for removal of cholesterol crystal could remove some other critical components such as liquid crystals, vesicles or proteins such as mucin and some low molecular weight glycoproteins which are also important in the nucleation process [Marianne et al, 1992]. Unlike native bile systems, model bile is a simpler and clear system containing three components viz: cholesterol, phospholipids and bile acids. Their association, dissociation during the cholesterol crystal nucleation process can be very well studied. Further the influence of any exogenous factor on the kinetics of cholesterol crystal nucleation can be evaluated.

The liver or bile duct may secrete pronucleating factors as it was found in T-tube bile or hepatic bile but gallbladder will not secrete these factors *per se* [Groen et al, 1988]. In the present study, hepatic biliary proteins from the spice (garlic and onion) and SO fed rats were examined for their influence on cholesterol crystal nucleation. Hepatic bile do not contain cholesterol crystals; thus the question of contamination is eliminated. Since the hepatic bile is very dilute and do not nucleate, it was concentrated to simulate gallbladder bile. During concentration no precipitation of any biliary components was observed. Concentration of hepatic bile by the gallbladder leads to lipid concentration. Upon concentrating the bile samples, there is an increase in vesicular C/PL ratio and an increased propensity to nucleate cholesterol crystals [Harvey et al, 1989]. Higher concentrations of components may lead to vesicular aggregation and vesicular aggregation precedes nucleation [Somjen and Gilat,1983]. Implication of bile concentration (dehydration) in the gallstone formation is important. As compared to hamsters and humans, there is an increased conversion of cholesterol to cholesteryl esters and to bile acids in rats. Therefore, the hepatic bile of rats seldom reaches supersaturation. However, in our

experiments, the high cholesterol diet caused a near saturated condition in bile, which was made supersaturated by dehydration. Earlier studies have shown a high cholesterol secretion in the bile of rats fed a high cholesterol diet and feeding garlic reduced the biliary cholesterol and increased bile secretion in rats [Platel and Srinivasan, 2000].

The addition of lithogenic group bile to the control group bile had a little effect on the nucleation time although the CSI of the mixture (50:50) was >1. Whereas, addition of SO and spice fed group bile even at the lowest proportion markedly increased the nucleation time of the lithogenic diet fed group. These differences in the nucleation time can be attributed to the presence of factors apart from supersaturation. These factors may be proteins as suggested by earlier reports [Burnstein et al, 1983; Lee et al, 1989]. It is suggested that lithogenic diet fed rats may secrete proteins into bile which act as promoters of cholesterol crystal nucleation whereas rats fed SO and spice secrete biliary proteins which act as antinucleators. An increase in the nucleation time of lithogenic bile upon addition of small quantity of SO and spice group bile shows that the antinucleating factor present in the SO and spices group probably neutralizes the pronucleating factors of the lithogenic bile and hence prolongs the nucleation time of lithogenic group bile. The bile of rats fed lithogenic diet probably contains large quantity of pronucleating activity but this pronucleating activity decreased upon feeding SO and spices. Thus feeding SO and spices decreased the pronucleating activity which suppressed the pronucleating activity.

Both high molecular weight and low molecular weight protein fractions separated by gel filtration from lithogenic group shortened the nucleation time indicating the presence of pronucleating factors. In contrast, the high molecular weight and low molecular weight protein fractions separated from SO and spice groups showed the presence of antinuleating factors which exerted a dose dependent effect.

The delipidation results in 30 – 40% loss of proteins from bile [Yamazki et al, 1988]. Hence, they were purified on affinity lectin column to examine whether sugar specificity of glycoproteins present in the bile of SO and spice fed groups would have any role in nucleation. A higher concentration of Con-A bound activity was isolated from lithogenic diet fed group bile. This glucose/mannose specific activity was shown to be a potent promoter of nucleation and this is in agreement with earlier reports. In the present study, this activity is associated with 24 KD protein as separated by electrophoresis. On the other hand in humans 130 KD protein has been shown to

have the activity [Lee et al, 1989]. Since the biliary affinity lectin binding proteins have been fully characterized their relevance to the data presented in this study is not complete. It is possible that the Con-A binding protein or its sugar moiety may interact with biliary lipids resulting in the formation of lipoprotein or glycolipid complexes [Afdhal et al, 1990]. Such interaction may cause destabilization of vesicles and micelles, the two major cholesterol carriers in the bile.

The present study is the first to report such a novel antinulceating activity in the hepatic bile of rats fed SO and spices (garlic and onion). This study provides evidence that the antilithogenicity of SO and spice is not only controlled by reducing hepatic and biliary cholesterol but perhaps also by causing the secretion of antinucleating proteins in the bile and by suppressing the pronucleating activity. There is no evidence that biliary proteins are affected by dietary components except indirectly by the fact that some bile acids stimulate secretion of mucin [O'leary et al, 1991] and a high cholesterol diet feeding leads to hypersecretion of mucin in praairie dogs [Lee et al, 1981].

In all the dietary groups both the cholesterol crystal promoting and inhibiting activities can be seen except for differences in their concentration. The concentration of promoting activity is higher in the lithogenic group than the inhibiting activity. This study shows that dietary components have a profound effect on the inhibiting or promoting activity. One possibility is that SO and spice feeding may reduce the promoting activity produced in response to the dietary cholesterol and may increase the synthesis or secretion of inhibiting activity.

This study clearly demonstrated that the liver or bile duct of rats secretes factors influencing the nucleation of cholesterol crystal. Feeding cholesterol in the diet increases the pronucleating factors. This release of pronucleating factors may be the result of the pathogenic effect of high cholesterol load on the liver. Feeding SO and spices (onion and garlic) increases the antinucleation factors which is the result of an antagonist effect of SO, garlic and onion. In this context, it is evident that feeding SO and spice (garlic and onion) prevent CGS not only by reducing cholesterol levels in bile and increasing bile acids, but also by increasing the antinucleation factors.

Lipid peroxides may also play a vital role in the CGS pathogenesis. Bile is a complex mixture rich in phospholipid, bile acids and cholesterol. But, little is known about the redox status of bile. Conceivably, biliary phospholipid may undergo peroxidation in conditions associated with oxidant stress. Peroxidation products may be factors

responsible for the cholesterol nucleation and thus leading to the formation of CGS. Despite increasing evidence of an important role of oxidant stress in various hepatobiliary diseases such as cholestasis [Ohshio et al, 1988], cholangiocarcinoma [Toyokuni, 1995], and liver transplantation [Galley et al, 1995], no attempt has been made to assess oxidant stress in bile. Earlier, Eder et al, (1996) have reported that generation of the lipid peroxidation products in model bile significantly reduced the cholesterol crystal formation time indicating that lipid peroxidation may play a role in CGS pathogenesis. *In vivo* study reports are also in the same lines where they have found that increased levels of MDA in the bile samples of patients with cholesterol gallstones as compared to stone free patients [Eder et al, 1995]. Our results are in agreement with the earlier reports, where LG group bile showed higher levels of MDA in normal and peroxidation induced conditions. But spices and SO could effectively reduce the levels of MDA formation in normal and peroxidation induced conditions leading to the delayed onset of nucleation of cholesterol in hepatic bile.

These observations indicated that SO and spice have a profound influence on promoting the antinucleating factors and inhibition of peroxidation in the bile. These factors help in stabilization of cholesterol carriers and keeping bile in solubilized form, which is thermodynamically feasible from inhibiting cholesterol from nucleating.

Chapter – VII

Summary and conclusions

- Sesame oil (SO) at 10 % level in the diet exhibited hypocholesterolemic effect both in normal and hypercholesterolemic rats
- SO reduced serum total cholesterol and this reduction was mainly occured in LDL+VLDL cholesterol fraction
- SO significantly reduced liver cholesterol by inhibiting HMG-CoA reductase activity
- SO also decreased the levels of serum and liver triglycerides
- SO significantly increased the cholesterol secretion into bile
- The denovo synthesis of cholesterol in animals was down regulated by SO feeding by inhibiting HMG-CoA reductase the rate limiting enzyme in cholesterol biosynthesis
- The hypocholesterolemic action of SO was brought about in animals by diminished absorption and enhanced excretion of cholesterol in feces and bile and decreased synthesis in liver
- SO was very effective in reducing the iron induced hepatotoxicity and lipid peroxidation in rats
- SO feeding reduced the peroxides by increasing the antioxidant enzyme activities in liver and serum
- The tocopherol content in the liver and serum was elevated by SO providing antioxidant defense cover
- The liver function marker enzymes in serum viz: ALAT, ASAT and LDH were significantly reduced by SO feeding exhibiting its hepatoprotective effect
- The fair sized clearly detectable CGS were induced in weanling mice fed lithogenic diet for 10 wks
- CGS score was significantly reduced in all the experimental groups compared to LG fed group
- The lowest incidence of CGS was occurred in HPO group

- Feeding garlic, onion and SO significantly decreased the biliary cholesterol as well as C/PL ratio
- The cholesterol saturation index (CSI) of bile in LG group was 1.9 and it was <1 in RG, HPG, RO, HPO and SO fed animals
- The % of CGS was correlated with cholesterol saturation index (CSI) irrespective of the diet
- The hydrophobicity index (HI) of bile was decreased significantly in spices and SO group compared to LG group
- The cholesterol degrading enzymes (sterol 27-hydroxylase and cholesterol 7α-hydroxylase) activities were increased by feeding SO and spices
- Spices and SO significantly reduced the serum and liver cholesterol and triglyceride content
- Heat processing of onion (boiling for 15 min) was more effective than raw onion in decreasing the incidence of CGS in mice. On the other hand, heat processing of garlic resulted in the reduction of antilithogenic activity compared to raw garlic
- The order of antilithogenic effect exhibited by spices and SO is: HPO>RG>SO>RO>HPG
- Regression of CGS at 5 and 10 weeks by spices and SO was studied in mice after establishing the presence of detectable CGS in gallbladder
- Spices and SO reduced the CGS score significantly at both time intervals, but more pronounced at 10 weeks in CGS prevailing conditions
- The regression of CGS occurred only in presence of experimental diets and not in the mice fed control diet alone
- The serum, liver and biliary cholesterol levels were decreased with prolonged feeding of spices and SO
- C/PL ratio in bile and other tissues was significantly decreased in spices and SO fed animals compared to LG diet
- During the regression studies, reduced CGS was well correlated with CSI in all the experimental animals

- Spices and SO increased HMG-CoA reductase activity. On the other hand, cholesterol degrading enzymes (cholesterol-7∝-hydroxylase and sterol-27-hydroxylase) activities were also increased during regression of CGS
- Hydrophobicity index (HI) and cholesterol saturation index (CSI) were decreased upon feeding SO and spices at both intervals during regression of CGS
- Bile from long-term fed mice (10 weeks) was subjected to gel permeation chromatography using sephadex-G-100 and the vesicles and micelles were separated
- Lithogenic bile from CGS prevailing mice resolved into three peaks viz: vesicular, small vesicular and micellar peaks. On the other hand bile from spices and SO fed groups decreased the relative peak areas in experimental groups
- The biliary lipid components of the vesicular fractions were redistributed and shifted to micellar fraction favoring solubilization of cholesterol in the bile by spices and SO feeding
- The potency of spices and SO in regressing the preformed CGS is: HPO>RG>SO>RO>HPG
- The lithogenic diet fed rat bile significantly decreased the cholesterol nucleation time. On the other hand spices and SO prolonged the cholesterol nucleation time
- In mixing experiments spices and SO fed rat bile significantly prolonged the nucleation time when incubated with lithogenic bile
- Feeding of spices and SO decreased the lipid peroxides in bile both in normal and induced conditions and enhanced the nucleation time of cholesterol in rat bile
- The hepatic bile of rats was subjected to gel permeation chromatography and high molecular weight (HMW) and low molecular weight (LMW) protein fractions were separated
- The HMW fractions from spices and SO group delayed the crystal growth, decreased the final crystal concentration and prolonged the nucleation time in model bile compared to LG group
- The LMW fraction from LG group shortened the crystal detection time whereas, fraction from spices and SO fed group bile delayed the crystal detection time in model bile

- The Con-A bound LMW protein fraction from LG group promoted the nucleation time and increased cholesterol crystal growth rate, whereas LMW fraction from experimental groups delayed the crystallization sequences and interfered with pronucleating activity

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Table 4.9: Effect of spices and SO on bile acid profile in bile during CGS induction in mice

Groups	TMC	TUDC	TC	TCDC	TDC	GC
mole (%) composition						
Control	0.24±0.07	0.021±0.005	0.59±0.06	0.048±0.009	0.056±0.005	0.02±0.005
LG	0.05±0.01[a]	0.008±0.002[a]	0.73±0.08[a]	0.040±0.012[a]	0.135±0.029[a]	0.02±0.009[a]
LG+RG	0.19±0.02[b]	0.015±0.005[b]	0.63±0.05[b]	0.045±0.01[b]	0.091±0.022[b]	0.02±0.003[b]
LG +HPG	0.15±0.02[b]	0.013±0.003[b]	0.65±0.09[b]	0.045±0.006[b]	0.111±0.09[b]	0.022±0.002[b]
LG +RO	0.19±0.03[b]	0.013±0.003[b]	0.62±0.11[b]	0.041±0.012[b]	0.104±0.03[b]	0.021±0.009[b]
LG +HPO	0.21±0.03[b]	0.015±0.005[b]	0.59±0.12[b]	0.038±0.013[b]	0.115±0.04[b]	0.018±0.007[b]
LG+SO	0.20 ±0.02[b]	0.014±0.005[b]	0.61±0.05[b]	0.044±0.01[b]	0.094±0.022[b]	0.017±0.003[b]

Values are mean ± SD of 6 samples / group, each sample constituting 3 mice
[a]Statistically significant when compared to control group at P< 0.01
[b]Statistically significant when compared to LG group at P< 0.01
Group legends are as in Table – 4.2
TMC-Tauromuricholic acid, TUDC-Tauroursodexoycholic acid, TC-Taurocholic acid, TCDC-Taurochenodexoycholic acid, TDC-Taurodeoxycholic acid, GC-Glycocholic acid.

Table 4.10: Effect of spices and SO on biliary fatty acid composition during CGS induction in mice

Dietary groups	16:0	16:1	18:0	18:1	18:2	20:4
Control	16.93±1.12	5.33±0.21	3.72±0.18	62.31±2.31	7.51±0.54	4.09±0.21
LG	25.25±1.89[a]	7.50±1.05[a]	3.38±0.14	47.17±3.21[a]	10.27±1.32[a]	4.04±0.21
LG+RG	25.85±2.31	7.68±1.56	3.46±0.23	48.30±3.13	10.52±0.89	4.14±0.32
LG+HPG	21.32±3.11	3.00±0.53[b]	1.84±0.14[b]	56.49±4.16[b]	9.24±0.98	8.08±0.65[b]
LG+RO	20.13±1.19[b]	2.84±0.14[b]	5.45±0.34[b]	55.97±4.16[b]	9.69±1.21	5.90±0.65
LG+HPO	22.14±2.01	3.21±0.31[b]	4.86±3.21	52.11±3.16[b]	8.78±1.65	8.90±0.31[b]
LG+SO	21.31±3.12	2.95±0.41[b]	3.98±0.22	48.65±3.59	8.95±1.11	5.45±0.61[b]

Values are mean ± SD of 6 samples/group, each sample constituting 3 mice
[a] Statistically significant when compared to control group at P< 0.01
[b] Statistically significant when compared to LG group at P< 0.01
Group legends are as in Table – 4.2

Table 5.7: Effect of short-term (5 weeks) feeding spices and SO on various bile salts in CGS prevailing mice

Dietary Groups	TMC	TUDC	TC	TCDC	TDC	GC
			mole fraction			
Control	0.27±0.04	0.02±0.001	0.59±0.004	0.04±0.001	0.05±0.003	0.02±0.0008
LG	0.05±0.004[a]	0.008±0.0005[a]	0.74±0.006[a]	0.04±0.002	0.12±0.002[a]	0.02±0.001
C	0.10±0.005[b]	0.006±0.0005	0.70±0.005	0.04±0.002	0.10±0.002	0.02±0.001
C+RG	0.19±0.004[b]	0.012±0.006[b]	0.64±0.008[b]	0.04±0.002	0.08±0.004[b]	0.02±0.001
C+HPG	0.14±0.004[b]	0.013±0.001[b]	0.63±0.02[b]	0.05±0.007	0.109±0.005[b]	0.022±0.0008
C+RO	0.19±0.001[b]	0.013±0.001[b]	0.58±0.014[b]	0.051±0.006[b]	0.12±0.009	0.026±0.005[b]
C+HPO	0.24±0.022[b]	0.013±0.002[b]	0.57±0.016[b]	0.044±0.003	0.11±0.02	0.018±0.0006
C+SO	0.17±0.004[b]	0.010±0.006	0.68±0.008[b]	0.04±0.002	0.08±0.004[b]	0.02±0.001

Values are mean ± SD of 6 samples / group, each sample constituting 3 mice
1sample = 3 mice.
[a] Statistically significant when compared to control group at $P < 0.01$
[b] Statistically significant when compared to LG group at $P < 0.01$
Group legends are as in Table –5.1
TMC-Tauromuricholic acid, TUDC-Tauroursodexoycholic acid, TC-Taurocholic acid, TCDC-Taurochenodexoycholic acid, TDC-Taurodeoxycholic acid, GC-Glycocholic acid.

Table 5.8: Effect of shor- term (5 weeks) feeding spices and SO on biliary fatty acid composition (%) in CGS prevailing mice

Dietary group	16:0	16:1	18:0	18:1	18:2	20:4
Control	18.92±1.25	4.98±0.85	3.72±0.54	63.52±8.52	6.89±0.58	1.96±0.11
LG	28.96±3.20[a]	4.86±0.89	3.56±0.25	49.85±6.5[a]	9.27±0.39[a]	3.54±1.01[a]
C	29.61±2.22	4.77±0.81	3.64±0.21	53.55±5.3	10.70±0.29	2.24±1.11
C+RG	23.85±2.74[b]	6.74±1.23	5.41±1.20[b]	53.62±3.45	8.4±1.52	1.98±0.85
C+HPG	23.2±2.22[b]	3.1±0.25[b]	1.68±0.23[b]	52.41±5.42	11.62±2.30	7.99±3.56[b]
C+RO	21.21±2.36[b]	3.56±0.53[b]	4.36±1.22	55.23±6.21	8.65±1.89	6.82±1.65[b]
C+HPO	19.65±1.89[b]	2.83±0.16[b]	3.96±0.92	58.14±3.49	8.21±0.87	7.21±0.31[b]
C+SO	23.35±1.56[b]	2.98±0.22[b]	3.25±0.12	56.62±3.21	8.52±0.83	5.26±0.36[b]

Values are Mean ± SD of 3 separate determinations.
[a] Statistically significant when compared to control group at $P < 0.01$
[b] Statistically significant when compared to LG group at $P < 0.01$
Group legends are as in Table –5.1

Table 5.16: Effect of long-term (10 - weeks) feeding spices and sesame oil on various bile salts in CGS prevailing mice

Dietary Groups	TMC	TUDC	TC	TCDC	TDC	GC
mole fraction						
Control	0.25±0.02	0.02±0.003	0.59±0.05	0.046±0.006	0.05±0.002	0.02±0.002
LG	0.05±0.006[a]	0.009±0.004[a]	0.73±0.0[a]	0.04±0.004	0.12±0.003[a]	0.02±0.003
C	0.15±0.004b	0.014±0.004b	0.69±0.02b	0.04±0.004	0.09±0.003b	0.02±0.003b
C+RG	0.20±0.006b	0.018±0.004b	0.63±0.007	0.03±0.003	0.08±0.007b	0.02±0.003
C+HPG	0.16±0.004b	0.016±0.002b	0.63±0.01b	0.05±0.01	0.123±0.02	0.02±0.001
C+RO	0.20±0.007b	0.013±0.001b	0.61±0.02b	0.035±0.009b	0.11±0.009	0.027±0.003
C+HPO	0.26±0.028[b]	0.015±0.008[b]	0.54±0.008[b]	0.043±0.004	0.11±0.02	0.017±0.002
C+SO	0.19±0.006[b]	0.014±0.004[b]	0.67±0.007[b]	0.03±0.003	0.08±0.007[b]	0.02±0.003

Values are mean ± SD of 6 samples / group, each sample constituting 3 mice
[a] Statistically significant when compared to control group at $P < 0.01$
[b] Statistically significant when compared to LG group at $P < 0.01$
Group legends are as in Table –5.1
TMC-Tauromuricholic acid, TUDC-Tauroursodexoycholic acid, TC-Taurocholic acid, TCDC-Taurochenodexoycholic acid, TDC-Taurodeoxycholic acid, GC-Glycocholic acid.

Table 6.1: Lipid composition, CSI, Nucleation time of the rat bile from various dietary groups

Dietary group	Lipids (mM)			Total lipid (g/dL)	CSI	Nucleation Time (days)
	BA	PL	Chol.			
Control	182.5±12.11	20.32±1.23	04.40±0.33	10.71±0.51	0.53±0.09	ND
LG	140.0±7.86[a]	25.63±2.05[a]	32.39±2.34[a]	10.11±0.43	2.16±0.23[a]	5
RG	173.2±6.00[b]	20.38±1.04[b]	15.94±2.01[b]	10.70±0.28	1.06±0.07[b]	15
HPG	152.4±15.70[b]	17.72±1.01[b]	20.46±2.88[b]	9.65±0.75[b]	1.30±0.08[b]	11
RO	150.9±12.40[b]	21.42±1.84[b]	16.85±1.76[b]	9.72±0.55	1.15±0.15[b]	14
HPO	175.7±11.04[b]	19.31±1.00[b]	12.45±1.80[b]	10.60±0.58	0.95±0.13[b]	18
SO	188.0±8.58[b]	20.23±1.56[b]	16.88±1.01[b]	11.72±0.39[b]	1.13±0.06[b]	15

Each value is the mean ± SD of 3 samples
ND – Crystals not detected even on 21 days
[a] Statistically significant when compared to control group at $P < 0.01$
[b] Statistically significant when compared to LG group at $P < 0.01$
Group legends are as in Table – 5.1