

EFFECTS OF INTERESTERIFIED FATS ON LIPOPROTEIN SUB FRACTIONS AND HEPATIC GENE EXPRESSIONS IN A HAMSTER MODEL

GOWRI NAGAPAN^{1*}; GOH YONG MENG²; NOOR LIDA HABI MAT DIAN¹; CHE ANISHAS CHE IDRIS¹; KANGA RANI SELVADURAY¹ and NAGENDRAN BALASUNDRAM¹

ABSTRACT

Palmitic rich interesterified (IE) fats exert detrimental effects on atherogenicity in animals but less significant effects in animal and human plasma lipids. Thus, it is important to investigate the role IE fats on lipid sub-fractions and hepatic gene expression involved in lipoprotein regulation. F1B male Golden Syrian hamsters (*Mesocricetus auratus*) were fed high-fat diets ad libitum containing 0.1% dietary cholesterol and 30.0% energy from dietary fat, either native or IE fats namely palm olein (PO), chemically IE palm olein (CIEPO), sal fat blend (SFB) and chemically IE sal fat blend (CIESFB) for 12 weeks. Plasma lipid profiles, low density lipoprotein (LDL) and high density lipoprotein (HDL) sub-fractions and hepatic gene expression levels were determined. PO and CIEPO fed hamsters had 38% and 27% higher plasma HDL levels compared to SFB and CIESFB, respectively. Animals given PO diet had greater proportion of the larger HDL particles than SFB and CIESFB fed animals ($p < 0.05$). Whereas, animals fed with SFB and CIESFB had greater proportion of larger LDL particles compared to both palmitic counterparts. All diets upregulated genes involved in liver fat accumulation such as CXCL16, VLDLR and APO E. SFB diet showed significant ($p < 0.05$) 16-fold upregulation in CXCL16 gene. Gene expression for ABCA1, APO A1 and CETP were upregulated all groups in response of reverse cholesterol transport (RCT). Palmitic-rich diets presented significant upregulation in APO A1 gene ($p < 0.05$). LDL metabolism related genes such as LDLR, PCSK9, APO B, CYP7A1, PCSK9 were downregulated in all diets. In conclusion, native and IE saturated high-fat diets, induce liver steatosis in hamsters as shown in CXCL16, VLDLR and APOE expression. In this condition, cholesterol clearance via RCT was activated with expression of related genes such as ABCA1, LCAT, APO A1 and CETP. However, these effects on plasma level HDL cholesterol and large HDL sub-fractions were only seen in palmitic rich fats. Whereas, LDLR mediated cholesterol clearance was downregulated with suppression of LDLR gene with similar effects on plasma LDL in all diets.

Keywords: hepatic genes, interesterified fats, lipoprotein sub-fractions.

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INTRODUCTION

Intesterified (IE) fats are one of the alternatives used in the food industry to reformulate and

reduce the levels of *trans* fatty acids. IE fats are produced using either chemical or enzymatic method by rearrangements of the fatty acids in the glycerol backbone (Berry, 2009). The interesterification process alters the triacylglycerol (TAG) composition and physical properties of the fats and results in the repositioning of saturated fatty acids (SFA) such as palmitic or stearic acids to the *sn*-2 position which does not normally occur in most natural triglycerides. Palm oil products are excellent alternatives for *trans*-fat in food formulations. Palm oil solid fractions naturally

¹ Malaysian Palm Oil Board,
6 Persiaran Institusi, Bandar Baru Bangi,
43000 Kajang, Selangor, Malaysia.

² Department of Veterinary Preclinical Sciences,
Faculty of Veterinary Medicine,
Universiti Putra Malaysia, 43400 UPM Serdang,
Selangor, Malaysia.

* Corresponding author e-mail: gowri@mpob.gov.my

have high melting point and possess improved oxidative stability compared to other oils. *Trans*-fat replacers can be produced through established palm oil modification techniques such as blending palm oil with other oils and interesterification with different fats to alter the physical properties and produce fats with improved functionality in various food applications (Parveez *et al.*, 2020). Palm olein (PO) which has a melting point of 13°C-15°C is predominantly (65%) having monosaturated oleic acid at the *sn*-2 position. Interesterification of PO produces solid palm fat with melting point of 33°C-35°C which is more suitable for food applications as bakery fats. It has been hypothesised that the increase in SFA at the *sn*-2 position may have metabolic effects as these 2-monoacylglycerols stay intact during digestion and absorption process (Berry 2009; Filippou *et al.*, 2014).

High SFA diets have been associated with elevated serum of low-density lipoprotein cholesterol (LDL-C) levels (Sacks *et al.*, 2017), which is a risk factor for the development of atherosclerosis and cardiovascular disease (FERENCE *et al.*, 2017). On the other hand, monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) have beneficial effects on blood lipid profiles (Mensink *et al.*, 2003; 2016). Different types of SFAs have different effects on lipid markers. Lauric, myristic and palmitic acids have plasma cholesterol raising effect while stearic acid has neutral effect (Mensink *et al.*, 2003; 2016).

Acute and chronic human trials have evaluated the effects of IE fats on cardiovascular risk and diabetes. The majority of the studies focused on the effects of IE fats on plasma lipid concentrations (Nestel *et al.*, 1995; Robinson *et al.*, 2009; Sanders *et al.*, 2011; Sundram *et al.*, 2007; Yli-Jokipii *et al.*, 2001). In healthy individuals, palmitic acid-rich IE fat has lowered postprandial plasma triglyceride (TG) and non-esterified fatty acids (NEFA) compared to palm oil and high-oleic sunflower oil counterparts (Sanders *et al.*, 2011). A chronic human trial on hypercholesterolemic men has reported that intake of high-palm oil margarine and its IE form has similar effects on plasma lipids (Nestel *et al.*, 1995). A recent review by Alfieri *et al.* (2018) provides an overview on the role of TAG structures and IE palmitic and stearic acid-rich fats on fasting and postprandial lipemia, focusing our attention on their physical properties and their effects on human health. There are very few data regarding the effects of randomised fatty acids on fasting plasma lipemia, however, they indicate that the consumption of meals with IE fats does not influence, or is able to reduce, fasting lipemic profiles compared to consumption of meals containing a mixture of native fats with the same fatty acids profile (Alfieri *et al.*, 2018).

In addition, data on the effects of randomised fatty acids on postprandial lipemia has shown

the reduction of postprandial plasma TAG concentrations, in young, adult and elderly subjects, with a more pronounced effect in women compared to age-matched men. In postprandial conditions, IE fat-rich meals could be able to reduce cardiovascular diseases risk. Nevertheless, as quoted above, plasma TAG concentrations and cholesterol fractions are not the only measurable factors related to cardiovascular diseases risk, and so other independent causes still need to be evaluated (Alfieri *et al.*, 2018). A recent work by Ng *et al.* (2018), has reported that IE palmitic acid diet had marginally induced adverse effects by raising body weight and body mass index (BMI) at week six and serum TAG, body fat percentage, and leptin concentrations at week 8. It was postulated that, these effects could have been due to greater fat absorption and lipogenesis in adipose tissue for the IE palm group, which suggests that the types and lengths (C16:0 and C18:0) of the fatty acids which are predominantly situated on the TAG molecule, may play an important influence on lipid metabolism (Ng *et al.*, 2018).

Several animal studies have investigated the effects of these SFA at *sn*-2 position on atherosclerosis development. However, the possible mechanisms involved in this process were not explored very much. Earlier studies by Kritchevsky *et al.* (1998; 2000) have reported that the IE fat adversely influenced the severity of atherosclerosis development in male New Zealand white rabbits but did not influence cholesterol and TG plasma concentrations (Kritchevsky *et al.*, 1998; 2000). Another animal study has reported that, a diet enriched with IE fats rich in SFA and PUFA showed a reduction in plasma lipids in Wistar rats, with an increase in low density lipoprotein (LDL) receptor and SREBP-2 gene expressions (Reena and Lokesh, 2007). The interesterification process did not alter plasma lipid concentrations, however, high-fat diet containing palmitic IE triggered hepatic fibrosis and adipocyte hypertrophy with inflammatory response in LDLr-KO mice (Lavrador *et al.*, 2019) and induce atherosclerosis development by promoting cholesterol accumulation in LDL particles and macrophagic cells, activating the inflammatory process in LDLr-KO mice (Afonso *et al.*, 2016).

The current investigation was undertaken to look in depth at the possible effects of native and IE palmitic acid- versus stearic acid- rich fats on lipoprotein sub-fractions and hepatic gene expression in a hamster model. The effects of IE saturated fat positioning in the TAG structure on atherosclerosis and lipid profiles are well established in animal models and humans. However, the role of these type of IE fats with the SFA at the *sn*-2 position on LDL and HDL sub-fractions and hepatic gene expression particularly

on underlying mechanisms on cholesterol metabolism are limited and therefore, need further investigation.

METHODS

Hamsters and Diets

Animal procedures carried out in this study were approved by Animal Care and Use Committee, Universiti Putra Malaysia, Serdang, Selangor, Malaysia (AUP No.: R078/2014). Forty, male specific pathogen free (SPF) male Golden Syrian hamsters (*Mesocricetus auratus*), [12-14 weeks-old: 110.2 ± 9.9 g, mean \pm standard deviation (SD)] obtained from Janvier laboratory, France were used for the trial. Two animals were housed per individually ventilated cage (IVC) at SPF room, at the Preclinical Research Facility, Malaysian Palm Oil Board (MPOB), Malaysia. The SPF room was maintained in a controlled environment ($18^\circ\text{C} \pm 1^\circ\text{C}$, 55% humidity) with a 12 hr-light/-dark cycle. Hamsters were fed a commercial diet (Altromin #1324 Maintenance Diet) from Altromin Spezialfutter GmbH and Co. KG, Germany for two weeks. Following acclimatisation, the hamsters were randomly assigned into four groups (n=10 animals per group) and fed *ad libitum* with customised commercial high-fat diet prepared and pelleted by Altromin International, Germany for 12 weeks. The high-fat diet contained 30% energy from test fat and 0.1% cholesterol to induce the development of atherosclerosis. The nutrient compositions of the treatment diets are presented in Table 1. The four experimental diet groups were classified as follows: PO, chemically interesterified palm olein (CIEPO), sal fat blend (SFB) and chemically interesterified sal fat blend (CIESFB) for 12 weeks. The PUFA content namely C18:2 has been standardised in all the test fats. Slip melting point (SMP) for IE test fats have been standardised below 40°C with lower solids for better digestibility. Food intake was recorded daily, and body weights were recorded weekly.

At the end of the experiment, the hamsters were anaesthetised with a mixture of ketamine [50 mg/kg body weight (wt.)] and xylazine (10 mg/kg body wt). Blood samples were collected by cardiac puncture and plasma was separated after centrifugation at 3000 g for 15 min at 4°C and kept at -80°C until analysis. Liver samples were collected, weighed, and one part of the tissue was immediately frozen in liquid nitrogen and kept at -80°C until analysis. Liver fat extraction was a modification of the Folch method (Folch *et al.*, 1957). Approximately 3-5 g frozen liver were minced and transferred into a conical flask. Then, 50 mL of chloroform-methanol (2:1, v/v) were

added, followed by a 2 min homogenisation and transferred to separating funnel. 40 mL of 0.9% (w/v) sodium chloride (NaCl) solution was added and samples were then shake for several minutes. The samples were left overnight and later the fat at the bottom layer was collected, dried, and weighed.

TABLE 1. NUTRIENT COMPOSITION OF EXPERIMENTAL DIET

Ingredients	Amount (g^{-1} kg)	% En
Experimental oils ¹	150.0	30.8
Casein	110.0	10.0
Lactalbumin	110.0	10.0
L-arginine	2.5	0.2
L-tryptophan	0.3	0.0
Corn starch	370.2	33.8
Dextrose	165.0	15.1
Cellulose	44.0	
Vitamin mix	10.0	
Mineral mix	35.0	
Choline bitartrate	2.5	
Cholesterol	1.0	

Note: ¹PO - palm olein; CIEPO - chemically interesterified palm olein; SFB - sal fat blend; CIESFB - chemically interesterified sal fat blend; customised commercial diets prepared and pelleted by Altromin International, Germany.
% En - percentage of energy.

Test Fat Preparation

PO (brand-Vesawit) was purchased from a local supermarket (Tesco Hypermarket). The CIEPO (IPO, iodine value 56), was purchased from Wilmar PGEO Edible Oils, Johor, Malaysia). Sal stearin was a generous gift from 3F Industries Hyderabad, India. Sal stearin was blended with high oleic sunflower oil in the ratio of 70:30 and subjected to chemical interesterification (CIE).

Chemical Interesterification (CIE)

CIE was performed based to the method reported by Lida *et al.* (2002). Oil was heated and dried for 30 min at 110°C under vacuum. Sodium methoxide (0.2%) was added to catalyse the reaction. After 60 min of stirring at a constant speed of 3000 rpm, the oil was then cooled to 60°C - 70°C . A citric acid solution (20%) was later added to deactivate the residual catalyst. The oil was then washed with excess hot water (70°C - 80°C) to remove soap by-products. Washing was repeated several times to

ensure that the sample was completely free from citric acid, catalyst residue and soaps formed by the reaction between sodium ion with any free fatty acids present or produced during the process. The IE oil was then dried under vacuum at 110°C for approximately 60 min. One percent bleaching earth was then added to the dried IE oil to lighten its colour. The oil and bleaching earth were left to react for 30 min at 90°C-100°C followed by cooling of the mixture to 60°C. The mixture was then filtered to separate the oil and the bleaching earth. The bleached IE oil was then refined to remove free fatty acids using short path distillation method at 240°C.

Determination of Fatty Acid Compositions (FAC) of Dietary Oils and Diets

The FAC of test fats was determined by converting fatty acids of TAG to fatty acid methyl esters according to AOCS Official Method (1998) with slight modification and analysed on a Perkin Elmer Autosystem GC (PerkinElmer, Inc., California, USA) using SGE Capillary BPX70 column (part No. 054602, SGE Analytical Science Pty. Ltd., Milton Keynes, United Kingdom).

From the data on the fatty-acid composition, the following were calculated: The index of atherogenicity (IA) was developed by Ulbricht and Southgate (1991), and characterises the atherogenic potential of FA. As the PUFA/SFA ratio is too general and unsuitable for assessing the atherogenicity of foods, Ulbricht and Southgate (1991) proposed a new index, IA, based on PUFA/SFA considering the available evidence, and then checked whether the resulting values were in accordance. The Equation for calculating IA is:

$$\text{Index of atherogenicity, IA} = [\text{C12: 0} + (4 \times \text{C14: 0}) + \text{C16: 0}] / \Sigma\text{UFA}$$

Determination of Fatty Acids in the *sn*-2 Position

The fatty acid esterified at the *sn*-2 position was determined according to lipase hydrolysis method using AOCS Official Method (1998). Exactly 0.5 g of sample was mixed with 100 mg of pancreatic lipase in 10 mL of tris-hydroxymethylaminomethane (Tris) buffer (pH 8.0) and mixed well. Then, 2.5 mL of bile salt (0.1% w/v) and 1.0 mL of calcium chloride (2.2% w/v) were added. The mixture was shaken well and incubated at 40°C for 1 min. One mL of hydrochloric acid and 1 mL of diethylether were then added to stop the reaction, followed by vigorous mixing by an electric shaker. Then, it was allowed to stand for separation. The bottom layer was removed, and the upper layer was spotted onto a thin-layer chromatography (TLC) plate which was then developed with hexane, diethyl ether and formic acid in proportions 70/30/1 (v/v/v). The

monoacylglycerol (MAG) formed was separated using TLC plate. Plate was air dried and sprayed with 2'7'-dichlorofluorescein solution. The MAG band was identified under ultraviolet light, scraped, methylated and analysed for the FAC using a gas chromatography (GC) equipment (Agilent, Santa Clara, USA) which was equipped with a flame ionisation detector. Helium was used as the carrier gas and the total gas flow rate was at 0.8 mL min⁻¹. The oven temperature was set initially at 130°C. This temperature was then increased to 190°C at 6°C min⁻¹ and later to 200°C. It was held isothermally for 2 min at 200°C.

Physico-chemical Analysis of Test Oils

The solid fat content (SFC) and SMP of the test fats were analysed by Analytical and Quality Development Unit, MPOB. The SFC and SMP of the test fats were analysed using MPOB Test Methods (Ainie *et al.*, 2004). SFC was evaluated using a pulsed Nuclear Magnetic Resonance (NMR) with a non-stabilised parallel procedure (Tarmizi *et al.*, 2008a). SMP was based from Tarmizi *et al.* (2008b).

Plasma Lipid Analysis

Total cholesterol (TC) and TG, high-density lipoprotein cholesterol (HDL) and low-density lipoprotein cholesterol (LDL) analyses were conducted using enzymatic assay kits (Roche Diagnostics GmbH, Mannheim, Germany), as per manufacturers protocol on the clinical chemistry autoanalyser, Roche/Hitachi 902.

LDL and HDL Sub-fractions Analysis

Cholesterol levels in the lipoprotein sub-fractions; very low density lipoprotein (VLDL), large buoyant LDL (IbLDL), small dense LDL (sdLDL), large HDL and small dense HDL (sdHDL) were measured using the quantimetrix lipoprint LDL system, a linear polyacrylamide gel electrophoresis method (Lee *et al.*, 2014). Exactly 25 µL of plasma sample was added to polyacrylamide gel tubes and followed by 200 µL of loading gel. Gel tubes were placed in the preparation rack and contents mixed by inverting the tubes for 6-8 times. Then, the gel tubes were photopolymerised for 30 min. After 30 min, the tubes were placed into the electrophoresis chamber. The top and bottom portions of the chamber were filled with electrophoresis buffer (tris-hydroxymethyl aminomethane 66.1 g/100 g, boric acid 33.9 g/100 g, pH 8.2-8.6). Electrophoresis was run for 60 min at 36 mV. Migration of the HDL fraction was observed 1 cm from the bottom of the gel tube. Gel tubes were rested for 30 min and scanned with a scanner.

Hepatic Gene Expression Analysis

Hamster liver mRNAs were isolated from liver samples and the expression levels for genes involved in cholesterol metabolism were estimated using bead-based multiplex assay as described by the manufacturer’s protocol (QuantiGene; Panomics/Affymetrix, California, USA) designed for Luminex type analytical platform. Samples were analysed with a Bio-Plex 200 System Array reader with Luminex 100 X-MAP technology. Data were analysed using Bio-Plex Data Manager Software Version 5.0 (Bio-Rad). Gene expression data were normalised relative to internal control and two housekeeping genes; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and glucuronidase beta (GUSB). The QuantiGenePlex 2.0 Plex Set is shown in Table 3.

Statistical Analyses

Statistical analyses were carried out by Graph Pad Prism software (version 6.00; Graph Pad La Jolla, CA 9203, USA). All data were checked for normality. One-way analysis of variance (ANOVA) followed by the *post-hoc* Bonferroni’s multiple comparison test were performed. Value of $p < 0.05$ was considered statistically significant. Data was presented as mean \pm SD.

RESULTS

Test Fat Composition

The total and *sn*-2 FAC and physiochemical properties of the test fats are shown in Table 2. Both native and IE fat had similar total FAC and

differed only in *sn*-2 FAC. The *sn*-2 proportion of palmitic acid in PO was 10.0% vs. 43.0% in CIEPO. As for SFB, the proportion of stearic acid was 12.0% vs. 39.0% in CIESFB. The PO and SFB were fully melted with lower solids of 2.3% and 1.5% at 37°C, whereas, the IE fats, CIEPO and CIESFB had 8.3% and 7.3% solids at 37°C, respectively. PO has SMP of 15°C, whereas CIEPO, SFB and CIESFB had SMP in the range of 35°C-38°C. The IA was higher in palmitic-rich test fats, IA: 0.77 compared to 0.08 in stearic-acid test fats. The IA indicates that the relationship between the sum of SFAs and the sum of unsaturated fatty acids (UFAs). The main classes of SFAs, which include C12:0, C14:0 and C16:0, with the exclusion of C18:0, are considered pro-atherogenic (they favour the adhesion of lipids to cells of the circulatory and immunological systems. UFAs are considered to be anti-atherogenic as they inhibit the accumulation of plaque and reduce the levels of phospholipids, cholesterol, and esterified fatty acids. Therefore, the consumption of foods or products with a lower IA can reduce the levels of TC and LDL-C in human blood plasma (Chen and Liu., 2020).

Food Intake and Body Weight

The total food intake, weight changes and weekly body weights are as shown in Table 4. All groups showed body weight losses with highest in PO followed by SFB, CIESFB and CIEPO groups. CIEPO group showed the lowest body weight loss. However, the results were not statistically significant. Food intake was highest in SFB followed by CIESFB, CIEPO and PO. However, no significant difference was noted. Liver weight was significantly lower in CIESFB compared to PO and CIEPO groups.

TABLE 2. FATTY ACID COMPOSITION, SFC, SMP AND IA OF THE TEST OILS

FAC (%)	PO		CIEPO		SFB		CIESFB	
	<i>sn</i> -1,2,3	<i>sn</i> -2						
C16:0	39.9	10.2	41.0	43.0	4.6	2.4	4.6	5.22
C18:0	4.4	1.6	4.3	4.2	34.0	12.2	35.0	38.8
C18:1	44.1	67.0	43.2	41.0	45.0	78.5	45.0	46.8
C18:2	10.4	19.0	9.9	10.0	10.5	5.0	10.4	3.1
C20:0	0.3	0.1	0.3	0.3	4.1	2.7	4.6	5.2
C18:3	0.2	0.3	0.1	0.1	0.2	0.2	0.2	0.1
SMP	15.3°C		35.4°C		35.1°C		38.3°C	
SFC	2.3		8.3		1.5		7.3	
Index of atherogenicity (IA)	0.73		0.77		0.08		0.08	

Note: FAC - fatty acid composition; IA - index of atherogenicity; SFC - solid fat content; SMP - slip melting point; PO - palm olein; CIEPO - chemically IE palm olein; SFB - sal fat blend; CIESFB - chemically IE sal fat blend.

TABLE 3. GENES OF INTEREST FOR HEPATIC GENE EXPRESSION ANALYSIS

Genes	Abbreviations	Accession numbers	Species
Chemokine (C-X-C motif) ligand 16	CXCL16	XM_005067827	Golden hamster
Low-density lipoprotein receptor isoform X2	LDLR	XM_005078537	Golden hamster
Stabilin 1	STAB1	XM_013111841	Golden hamster
Apolipoprotein E	APOE	XM_005086320	Golden hamster
Very low-density lipoprotein receptor	VLDLR	XM_013110512	Golden hamster
Proprotein convertase subtilisin/kexin type 9	PCSK9	XM_013114871	Golden hamster
Apolipoprotein A1	APOA1	NM_001281657	Golden hamster
Apolipoprotein B-100	APOB	XM_005079084	Golden hamster
ATP-binding cassette sub-family A member 1	ABCA1	XM_005076485	Golden hamster
Phosphatidylcholine-sterol acyltransferase	LCAT	XM_005076352	Golden hamster
Apolipoprotein D	APOD	XM_003495384	Chinese hamster
Scavenger receptor class B member 1 isoform X4	SCARB1 (SRB1)	XM_013120965	Golden hamster
Cholesteryl ester transfer protein	CETP	M63992	Golden hamster
Cholesterol 7-alpha-monooxygenase	Cyp7a1	XM_016972392	Chinese hamster
Acetyl-CoA acetyltransferase 1	ACAT1	XM_005069541	Golden hamster
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH	U10983	Golden hamster
Hypoxanthine-guanine phosphoribosyltransferase	HPRT	XM_005085546	Golden hamster
Glucuronidase, beta	GUSB	XM_013120748	Golden Hamster

TABLE 4. GROWTH PARAMETERS IN HAMSTERS AFTER INTAKE OF EXPERIMENTAL DIETS FOR 12 WEEKS

Group	PO	CIEPO	SFB	CIESFB
Weight changes (g)	-16.94 ± 7.14	-12.56 ± 7.75	-16.58 ± 10.21	-16.39 ± 4.92
Total food intake (g)	889.77 ± 186.48	862.52 ± 171.92	984.37 ± 232.49	892.53 ± 195.35
Liver				
Liver weight (g)	10.33 ± 0.92a	10.25 ± 1.09a	9.33 ± 1.73	8.30 ± 0.91b
Liver fat content (per 100 g)	19.41 ± 7.62	23.98 ± 1.84	19.47 ± 5.75	22.93 ± 3.24

Note: PO - palm olein; CIEPO - chemically IE palm olein; SFB - sal fat blend; CIESFB - chemically IE sal fat blend.

TABLE 5. PLASMA LIPIDS, HDL AND LDL SUB-FRACTIONS

Parameters	PO	CIEPO	SFB	CIESFB
Lipid profile (mg/dL)				
TC	487.66 ± 76.69	456.3 ± 83.4	426.03 ± 53.23	435.89 ± 50.57
TG	116.8 ± 29.64	104.3 ± 24.91	98.08 ± 24.49	91.40 ± 29.96
LDL	222.53 ± 96.52	204.67 ± 56.99	226.82 ± 71.99	228.21 ± 54.2
HDL	292.66 ± 16.52a	268.61 ± 40.78a	230.41 ± 34.02b	255.23 ± 26.53b
TC/HDL ratio	1.67 ± 0.28	1.71 ± 0.27	1.89 ± 0.39	1.72 ± 0.20
LDL sub-fractions (%)				
VLDL	14.53 ± 2.47	15.46 ± 1.55	15.22 ± 2.04	15.84 ± 0.53
Large LDL	8.98 ± 3.33a	9.97 ± 3.84a	15.08 ± 4.63b	13.19 ± 3.26b
sd LDL	1.45 ± 2.02	0.91 ± 0.56	2.24 ± 2.03	1.79 ± 1.55
Mean LDL size (nm)	268.6 ± 4.427	269 ± 3.33	268.8 ± 4.71	268.4 ± 4.4
HDL sub-fractions (%)				
Large HDL	44.9 ± 7.58a	43.27 ± 3.42ab	36.95 ± 5.79b	36.07 ± 4.26bc
sd HDL	1.77 ± 0.88	2.19 ± 1.21	2.83 ± 0.92	2.26 ± 1.36

Note: HDL - high density lipoprotein; LDL - low density lipoprotein; sd HDL - small dense high-density lipoprotein; sd LDL - small dense low-density lipoprotein; TC - total cholesterol; TG - triacylglycerol; VLDL - very low-density lipoprotein; PO - palm olein; CIEPO - chemically IE palm olein; SFB - sal fat blend; CIESFB - chemically IE sal fat blend.

n=10, Values are means ± SD; Means with unlike alphabets indicate a significant difference of $p \leq 0.05$.

Plasma Lipids, LDL and HDL Sub-fractions

Plasma lipid profile, LDL and HDL sub-fractions are as shown in *Table 5*. The PO group presented greater HDL cholesterol compared to SFB and CIESFB groups. Other lipid parameters were similar in all groups.

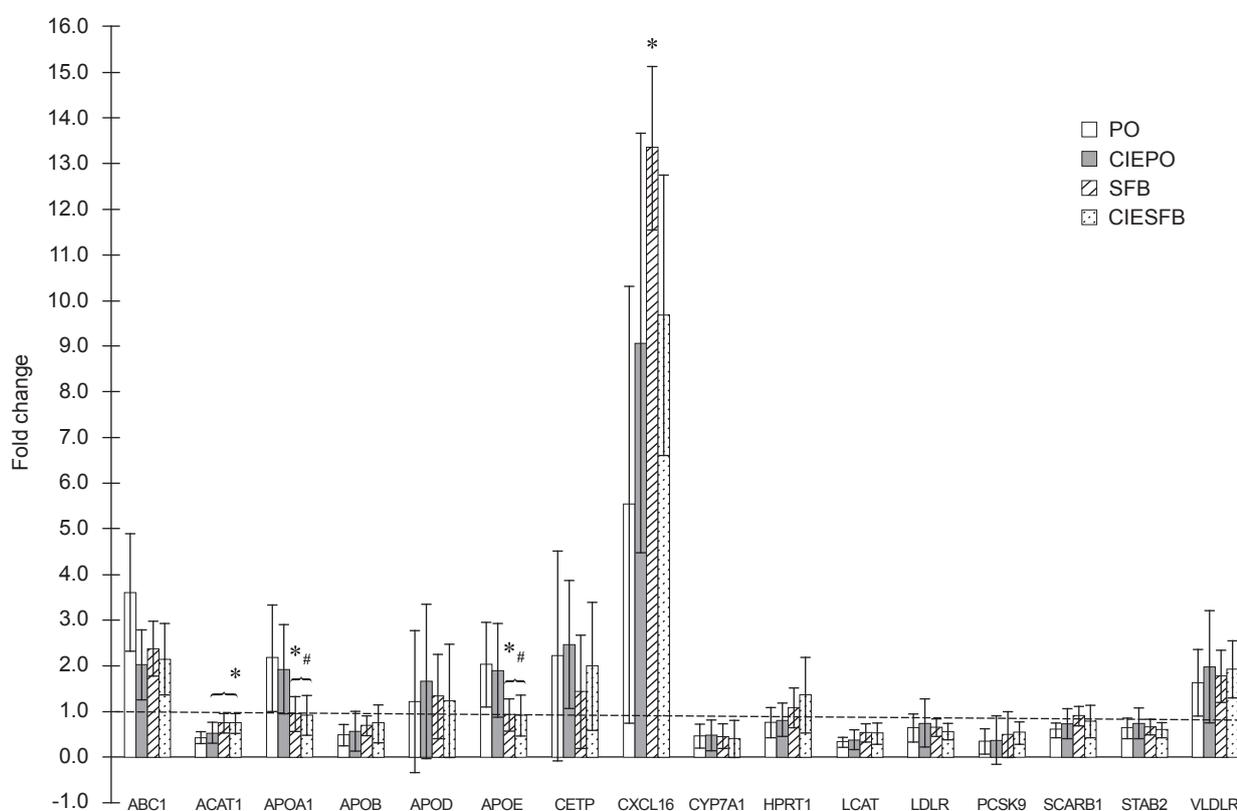
Animals fed with PO and CIEPO diets had increased proportion of larger HDL particles compared to stearic acid rich native and IE fats fed animals ($p < 0.05$). Plasma small dense HDL sub-fractions were similar in all groups. Large LDL sub-fractions were found increased in SFB and CIESFB fed animals compared to palmitic rich fat groups. However, the interesterification processes did not show any significant impact in all these lipoprotein particles levels.

Effects of *sn*-2 Palmitic vs. Stearic Acid on Hepatic Gene Expressions

Hepatic gene expression levels are depicted in *Figure 1*. Genes involved in cholesterol metabolism such Chemokine (C-X-C motif) ligand 16 (CXCL16), ATP-binding cassette sub-family A

member 1 (ABCA1), apolipoprotein A (APO A1), apolipoprotein E (APOE), cholesteryl ester transfer protein (CETP) and very low-density lipoprotein receptor (VLDLR) were upregulated. CXCL16 gene was found highly expressed all diets and significant differences seen (13 fold, $p < 0.05$) in SFB compared to PO. CXCL16 is often involved in inflammation-mediated lipid accumulation in liver. This finding was confirmed with signs of hepatic steatosis which was observed in all groups as shown in *Figure 2*. Significant group differences found in other genes such as APOE (PO vs. SFB, CIESFB and CIEPO vs. SFB, CIESFB $p < 0.05$); APO A1 (PO vs. SFB, CIESFB and CIEPO vs. SFB, CIESFB, $p < 0.05$).

Gene expression of acetyl-CoA acetyltransferase 1 (ACAT), apolipoprotein B (APOB), phosphatidylcholine-sterol acyltransferase (LCAT), low-density lipoprotein receptor isoform X2 (LDLR), proprotein convertase subtilisin/kexin type 9 (PCSK9), scavenger receptor class B member 1 isoform X4 (SCARB/SRB1) and stabilin 1 (STAB1) were down-regulated showing lower fold change than 1 with no group effects. Significant group differences were found in expressions of ACAT (PO vs. SFB, CIESFB $p < 0.05$).



Note: The gene expression data was normalised to two housekeeping genes (GAPDH, GUSB). The genes fold change was calculated by dividing the normalised value for the experimental samples PO - palm olein; CIEPO - chemically IE palm olein; SFB - sal fat blend; CIESFB - chemically IE sal fat blend by the normalised value of internal control. n=10, values are means \pm SD; * $p < 0.05$ vs. PO group; # $p < 0.05$ vs. CIEPO group.

Figure 1. Hepatic gene expression.

DISCUSSION

The main objective of this study was to evaluate the effects of IE palmitic and stearic-rich high-fat diets with added cholesterol on lipid profiles, lipoprotein sub-fractions and hepatic gene expression in hamster model. From the obtained results, 12 weeks high-fat diets with added cholesterol intake resulted in upregulation CXCL16 gene in all diets with 13 fold significant upregulation in SFB group in comparison with PO group. CXCL16 gene expression is often associated with fatty liver which was observed as pinkish liver in the hamsters. It has been reported that CXCL16 is involved in the pathogenesis of inflammatory diseases, such as atherosclerosis, cancer and kidney diseases (Barlic *et al.*, 2009). In our study, both saturated fats in native and IE form in high-fat scenario may have detrimental effects as seen in the gene expression levels of CXCL16 which was significantly increased causing inflammatory stress suggesting that the CXCL16 pathway may be involved in the development of fatty liver in hamsters.

Beside to the high expression of CXCL16, another gene often associated to fatty liver is VLDLR. VLDLR is a member of the LDLR family and is involved in lipid transport via apolipoprotein recognition. In VLDLR-deficient mice, blood TG concentration is high and increased lipid uptake into adipose tissue and demonstrating that VLDLR has a function in the uptake of TG-rich VLDL into peripheral tissues (Oshio *et al.*, 2021). VLDLR is highly expressed in tissues with active lipid metabolism, such as the heart, skeletal muscle, and adipose tissue (Tiebel *et al.*, 1999). The expression of VLDLR in the liver has been demonstrated to be lower than that in other organs. However, hepatic VLDLR gene expression is induced during endoplasmic reticulum (ER) stress, as well as fatty liver, and both of these inductions disappear in VLDLR-deficient and APO-E deficient mice (Jo *et al.*, 2013). We found that all high-fat diets induce upregulation of VLDLR and APOE it is strongly suggested that VLDLR-mediated lipid uptake into the liver may be a major cause of hepatic fat accumulation during ER stress. However, plasma TG levels were not affected in this study.

We found that both native and IE oils, namely PO and CIEPO diets beneficially elevated plasma HDL cholesterol levels regardless of the positioning of palmitic acid in TAG backbone compared to the stearic acid rich diets. HDL is the smallest lipoprotein involved in scavenging of excess cholesterol from peripheral tissues to liver via RCT. We next quantified the HDL sub-fractions by the Lipoprint gel electrophoresis system. Larger HDL particles were found to be increased in PO and CIEPO diets compared to stearic acid-rich diets.

The increases in both plasma HDL and HDL particle size in PO and CIEPO diets, were further confirmed with the expression of ABCA1 and CETP genes. Intake of palmitic and stearic-rich SFA diets resulted in upregulation of ABCA1 and CETP genes in liver. These observations may be due to the high cholesterol loading from the diets in which excess cholesterol is removed from arterial cells and sent to the liver for excretion into the bile acid via RCT which is mediated by HDL (Zelcer and Tontonoz, 2006). RCT promotes removal of excess cholesterol from arterial wall macrophages to the liver for conversion to bile acids and subsequent excretion in faeces (Zelcer and Tontonoz, 2006). A study has evaluated the effects of saturated fat on RCT in mice with similar results in HDL levels, but in this case the authors did not find differences in the rate of macrophage RCT (Escolà-Gil *et al.*, 2011; O'Reilly *et al.*, 2016). HDL confers atheroprotection by protecting arterial wall from atherosclerotic plaques formation (Borén and Williams, 2016). APO A1 (70% of the protein content of HDL) interacts with ABCA1 receptors in various cell types (hepatocyte, enterocytes and macrophages) to remove excess phospholipids and cholesterol to APO A1. This process results in the formation of nascent HDL particles (pre- β HDL), which can subsequently interact with Scavenger receptor class B member 1 (SR-B1) and ATP-binding cassette, sub-family G, member 1 (ABCG1), with the purpose of incorporating more cholesterol, forming a mature molecule of HDL (α -HDL). These processes are catalysed by the enzyme Lecithin-cholesterol acyltransferase (LCAT) (Marques *et al.*, 2018; Shen *et al.*, 2018). LCAT deficiency is associated with severely reduced concentrations of HDL and APO A1, whereas transgenic animals overexpressing LCAT show markedly higher plasma HDL and APO A1 levels (Huang *et al.*, 2016). In the present study, LCAT activity in hamsters fed both palmitic and stearic-rich diets were found suppressed. A number of studies showed a relationship of LCAT expression with the expression of APO A1. Incubation of hepatocytes with cytokines or injection of endotoxin in rats simultaneously reduced the plasma concentration of APO A1 and LCAT activity. On the other hand, there is no reduction in hepatic APO A1 mRNA (Rudling *et al.*, 2002), suggesting that the two genes may not be coordinately regulated. Plasma HDL cholesterol concentration is positively associated with the concentration of APO A1; thus, APO A1 gene expression may be an important determinant of HDL cholesterol levels (Getz and Reardon, 2017). In humans, SFA rises APO A1 protein and HDL cholesterol concentration, while PUFA decreased HDL cholesterol concentrations (Dorfman *et al.*, 2005; Hatahet *et al.*, 2003; Lichtenstein *et al.*, 1999; Plump, 1994). In contrast to the previous

studies, our findings showed that hepatic APO A1 gene expression was higher in both palmitic rich diets with concomitant increase in plasma HDL cholesterol and large HDL sub-fractions with suppression of LCAT gene. This finding is in agreement to an earlier work which reported SFA intake lowered LCAT activity in hamsters (Dorfman *et al.*, 2005; Fungwe *et al.*, 1998).

Scavenger receptor class B type I (SRB1) gene was found downregulated in palmitic-rich diets which maybe became responsible for the elevation of plasma HDL and their sub-fractions particularly the large HDL particles. Hepatic SRB1 is an HDL receptor that plays a role in determining and circulating levels of HDL. Overexpression of SRB1 is associated with lower concentrations of HDL cholesterol and associated with an increased risk of CVD (Fungwe *et al.*, 1998; Spady *et al.*, 1999). SRB1 increases HDL mediated transport of cholesteryl ester from peripheral tissues to the liver and reducing the cholesteryl ester transfer protein (CETP)-mediated transfer of cholesteryl ester to apoB-containing lipoproteins. It was reported that, hepatic mRNA levels of SRB1 were elevated in hamsters fed PUFA diets compared to SFA (butter and coconut oil) and *trans* fatty acids (Dorfman *et al.*, 2005). Dietary fatty acids have an impact on SRB1 which regulates the circulatory pool of cholesterol. Although SFA-rich diet has beneficial effects on HDL cholesterol concentrations, palmitic acid rich fats might have more beneficial effects compared to stearic acid- rich diets in the metabolism of HDL. CETP facilitates the exchange of cholesteryl ester and TG between lipoproteins from HDL through action of LCAT to LDL which subsequently contributes to the plasma LDL levels. Although CETP gene was found upregulated in all diets, however the LCAT expression was found downregulated with no effects seen on the plasma LDL levels in all diets.

With regards to LDL metabolism, it was found that intake of both stearic-rich diets had elevated large LDL particles with the downregulation of LDLR gene. LDLR found in liver is responsible for the removal of plasma LDL and lowers plasma cholesterol concentrations. LDLR is an important marker of atherosclerosis (Haerer *et al.*, 2012; Notarnicola *et al.*, 2010). Human studies have also shown that nearly 60%-70% of the excess plasma cholesterol is transported via LDL-receptor mediated uptake and HDL-mediated RCT (Goldstein and Brown, 2009; Han *et al.*, 2012). Under normal conditions, LDL is removed from circulation mostly by LDLR mediated liver uptake so that LDL cholesterol levels are stable in plasma. The absence of LDLR causes LDL to accumulate in plasma and removal is totally disrupted. Increased level of LDLR expression will result in reduction of serum LDL cholesterol levels by enhancing the uptake

and removal of LDL cholesterol (Reena *et al.*, 2011). In this study, high-fat diet induced suppression in the LDL receptor, which mediated clearance of LDL particles in all diets in hamsters.

As shown in previous studies (Afonso *et al.*, 2016; Kritchevsky *et al.*, 1998; 2000), positioning of palmitic and stearic in *sn*-2 position with interesterification process did not modify the plasma lipid concentrations, as seen between PO *vs.* CIEPO and SFB *vs.* CIESFB diets fed hamsters. Both SFAs showed similar effects in lipids profiles namely TC, LDL, TC/HDL ratio and TG. Further analysis with LDL sub-fractions, showed that stearic acid rich diets have large LDL particles. These findings are in agreement with a recent study in LDLr-KO mice by Afonso *et al.* (2016). Both palmitic and stearic acid diets had normal patterns of LDL and HDL sub-fractions, consisting of primarily larger and buoyant with less small dense particles.

CONCLUSION

Our findings suggest that, native and IE saturated high-fat diets, had induced liver steatosis in hamsters as shown from the expression of CXCL16, VLDLR and APOE genes that induced hepatic fat accumulation. In this condition, cholesterol clearance via RCT was activated with expression of related genes such as ABCA1, LCAT, APO A1 and CETP. However, these effects on plasma levels of HDL cholesterol and large HDL sub-fractions were only observed in hamsters fed with palmitic rich fats. Whereas LDLR mediated cholesterol clearance was downregulated with suppression of LDLR gene, with similar effects on plasma LDL in all diets.

The Limitations of the Study

There are some limitations of the present study which should be noted. For instance, although hepatic steatosis was observed, no further histological, markers of liver injury and inflammation analysis were conducted to identify the severity of the hepatic steatosis induced by the tested diets. Therefore, further studies are warranted in this context. Besides that, plasma levels of CETP and LCAT were not determined in this study. The strength of our study relies on the use of hamsters as a model, which is widely accepted as a suitable animal model for studying human cholesterol metabolism. The lipid profiles and susceptibility to dietary cholesterol of the golden Syrian hamster (*Mesocricetus auratus*) are similar to those of human (Kris-Etherton and Dietschy, 1997). In human and the hamster, LDL is the dominant lipoprotein, whereas HDL is the major plasma lipoprotein in other animal models

such as mouse, rat and monkey (Huang *et al.*, 2016). The hamster also exhibits similar CETP activity as man, which is absent in the rat (Huang *et al.*, 2016).

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