1,3-DIPALMITOYL-2-OLEOYLGLYCEROL, 1,3-DISTEAROYL-2-OLEOYLGLYCEROL AND TRIOLEOYLGLYCEROL DO NOT DIFFER IN THEIR EFFECTS ON POSTPRANDIAL LEVELS OF PLASMINOGEN ACTIVATOR INHIBITOR-1 AND MARKERS OF INFLAMMATION IN HEALTHY MALAYSIAN ADULTS

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ABSTRACT

The 1,3-dipalmitoyl-2-oleoylglycerol (POP), 1,3-distearoyl-2-oleoylglycerol (SOS) and trioleoylglycerol (OOO)-type of fats have different melting characteristics that may affect postprandial haemostatic and inflammatory marker concentrations. We tested the hypothesis that the predominance of either palmitic acid (16:0), stearic acid (18:0) or oleic acid (18:1) at the sn-1 and sn-3 positions of edible fats has different effects on postprandial haemostatic and inflammatory responses. Each of the 36 healthy adults (18 males, 18 females) received three different test muffins [each containing 53 g of test fat, i.e. *palm mid-fraction (PMF; POP-rich), shea stearin (SS; SOS-rich) or high-oleic sunflower oil (HOSF; OOO-rich)] on different mornings in random order separated by two weeks. During a postprandial test, each subject was provided with a test muffin plus a low-fat milkshake (total 3.67 MJ or 876 kcal) in the morning and blood samples were collected at half-hourly intervals until 4.0 hr. Overall, no significant difference ($p>0.017$) was observed between the three test meals for postprandial responses in plasma PAI-1, interleukin-6 (IL-6) and tumour necrosis factor–α (TNF-α) levels. The POP-SOS and OOO- type of tests fats induced similar postprandial responses in haemostatic and inflammatory markers measured in the present subjects.*

Keywords: fatty acids, postprandial PAI-1, IL-6, TNF-alpha.

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INTRODUCTION

Haemostasis is the process that ensures the integrity of the vascular system and involves a complex system of factors which normally form and degrade blood clots that work within a delicate balance. Emerging evidence suggests that some haemostatic [Factor VII (FVII) and PAI-1] and inflammatory markers [interleukin-6 (IL-6) and tumour necrosis factor – α (TNF-α)] are associated with increased risk of cardiovascular disease (CVD). Accumulating evidence also suggests that there is a relationship between dietary fatty acids and emerging haemostatic CVD risk factors (Lefevre *et al*., 2004).

The PAI-1, a key regulator of fibrinolysis, is the major physiological inhibitor of the plasminogen activators in the circulation system and thereby the principal inhibitor of the fibrinolytic system (Brazionis *et al*., 2010). Small changes in plasma PAI-1 can have

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significant effects on overall haemostasis. Studies conducted in regards to postprandial triglyceridemia have suggested that it increases plasma PAI-1 levels which would further strengthen the chances of thrombotic occlusion of a vessel after rupture of an atherosclerotic plaque (Duttaroy, 2005). Elevated PAI-1 activity has also been shown to be associated with elevated fasting plasma triacylglycerol (TAG) concentrations, obesity and the insulin resistance syndrome (Duttaroy, 2005; Brazionis *et al*., 2008).

Markers of inflammation have been implicated as risk factors for several degenerative diseases. Evidences from various studies conducted have suggested that markers of inflammation are strong predictors of CVD such as myocardial infarction and stroke. Baer *et al*. (2004) pointed out that these markers include proinflammatory cytokines [*e.g*. interleukin 6 (IL-6)], cell adhesion molecules (CAM-*e.g*. selectins), and acute phase proteins [*e.g*. C-reactive protein (CRP)].

The positional distribution of fatty acids in the TAG molecule varies greatly among fats and oils of different origin. In the food industry, there is a great demand for edible fats with different forms of TAG which can serve as a cocoa butter equivalent (CBE), *i.e*. as an important alternative for chocolates and other confectionary products. Cocoa butter consists of three main TAG species - POP, SOS and 1(3)-palmitoyl-3(1)-stearoyl-2-oleoylglycerol (POS). It was noted that low-price commercial oils that have TAG with predominantly oleic acid in the sn2-position, such as palm oil (as palm-mid fraction- POP-type TAG), illipe (POS-type TAG) and shea (as shea stearin-SOS type TAG) can serve as a CBE (Ong and Goh, 2002). In fact, palm oil, illipe and shea are listed in the European Chocolate Directive 2000/36EEC as CBE.

Therefore, it is important to find out if a CBE such as palm mid-fraction (PMF) has similar effects as shea stearin on postprandial haemostatic and inflammatory responses. This forms the research question as well as the justification for the present study.

MATERIALS AND METHODS

Subjects

A total of 36 healthy adults (18 males and 18 females, aged 25–50 years) were recruited to participate in this study. All volunteers underwent a health screening which included: a) an abbreviated medical/physical examination [blood pressure, weight, height, body mass index (BMI)], b) serum lipid profiling, c) plasma glucose determination, d) liver function tests (SGOT and SGPT), and e) kidney function test (serum creatinine). The inclusion criteria for this study were BMI 18.5 – 25.0 kg m^{-2}, systolic pressure <140 mm Hg, diastolic pressure <90 mm Hg, fasting total cholesterol <6.2 mmol $litre^{-1}$ (<240 mg $decilitre^{-1}$), fasting TAG <1.70 mmol $litre^{-1}$ (<150 mg $decilitre^{-1}$), fasting glucose 4.0 – 7.0 mmol $litre^{-1}$. Exclusion criteria for this study were individuals on cholesterol or blood glucose medication, substance abuse (*e.g*. alcohol, cigarette smoking), going overseas during the period of study, having blood clotting problem and for women, pregnant or lactating.

This study was approved by the Research and Ethics Committee, International Medical University, Kuala Lumpur, Malaysia in 2012 and this trial was registered at clinicaltrials.gov.my as NCT01428960.

Study Design

A randomised, double-blind cross-over (3 × 3 arms) orthogonal Latin-square design was used. Blood collections were conducted according to the the study protocol at every 30 min interval. Each subject received three experimental test meals in random order, two weeks apart, over a six-week period. Subjects were randomly allocated to one of six treatment sequences (ABC, BCA, CAB, ACB, CBA, or BAC). Each test meal consisted of a muffin and a milkshake and provided 3.67 MJ (875.6 kcal), 16 g protein, 83 g carbohydrate, and 53 g test fat. The % energy of fat, carbohydrate and protein in the test meals were 55% kcal, 38% kcal and 7.3% kcal, respectively and is common for the composition of test meals in an acute study (Cortes *et al*., 2006), which does not represent the macronutrient composition of the typical Malaysian diet.

Sample size calculations were based on 90% power at $p<0.017$ for cross-over within group comparison to detect an effect size of 0.5-SD unit for the primary outcome - plasma PAI-1 concentrations, which gave a sample size of 36 subjects after allowing for 10% dropout. Secondary outcomes were changes in IL-6 and TNF-α levels. The characteristics of the study participants are shown in *Table 1*.

Test Fats

The three test fats were PMF (IV 34.9) and SS (IV 34.1) which were obtained from Wilmar PGEO Edible Oils Sdn Bhd while HOSF was obtained from Intercontinental Specialty Fats Sdn Bhd, Selangor, Malaysia PMF and SS were blended with a small amount of sunflower oil (Mazola, Switzerland) so that linoleic acid (18:2,ω-6) was standardised across the meals at 7% kcal. The fatty acid composition of the experimental fats is shown in *Table 2*. PMF contained a similar proportion of saturated fatty acids (SFA) (62.7% *vs*. 62.6%) and oleic acid (33.7% *vs*. 33.0%) when compared to SS but contained more palmitic acid (57.1% *vs*. 1.8 %) and less stearic acid (5.0% *vs*. 60.8%). The test fats were incorporated into muffins, labelled with a code, and stored frozen until consumed.

TABLE 1. CHARACTERISTICS OF THE STUDY PARTICIPANTS

Variables	Women (n=18)	Men (n=18)	Mean ± SD
Age (yr)	23.0 ± 1.1	23.0 ± 1.9	23.3 ± 1.5
Weight (kg)	52.0 ± 5.6	63.1 ± 7.6	57.5 ± 8.7
Height (cm)	160.0 ± 4.2	172.0 ± 5.7	166.0 ± 8.0
BMI (kg m^{-2})	20.3 ± 1.6	21.3 ± 2.0	20.8 ± 1.8
Systolic BP (mm Hg)	111.0 ± 6.1	122.0 ± 10.0	116.0 ± 9.9
Diastolic BP (mm Hg)	73.0 ± 5.0	75.0 ± 7.6	74.0 ± 6.4
Total cholesterol (mmol $litre^{-1}$)	4.9 ± 0.7	4.4 ± 0.8	4.7 ± 0.8
HDL-chol (mmol $litre^{-1}$)	1.7 ± 0.3	1.4 ± 0.3	1.5 ± 0.3
LDL-chol (mmol $litre^{-1}$)	2.9 ± 0.6	2.7 ± 0.7	2.8 ± 0.6
TAG (mmol $litre^{-1}$)	0.7 ± 0.3	0.7 ± 0.4	0.7 ± 0.3
Fasting glucose (mmol $litre^{-1}$)	4.6 ± 0.6	4.7 ± 0.3	4.6 ± 0.3

Note: BMI - body mass index, BP - blood pressure, HDL - high-density lipoprotein, LDL - low-density lipoprotein, TAG - triacylglycerol. All values are as mean ± SD.

TABLE 2. FATTY ACID COMPOSITION OF THE TEST FATS

Fatty acid	PMF (% w/w)	SS (% w/w)	HOSF (% w/w)
C14:0	0.61	ND	ND
C16:0	52.85	2.40	4.44
C18:0	4.87	57.20	2.41
C20:0	ND	ND	ND
C24:0	ND	ND	ND
SFA	**58.33**	**59.60**	**6.85**
C16:1	ND	ND	ND
C18:1	33.73	32.29	85.58
MUFA	**33.73**	**32.29**	**85.58**
C18:2	7.93	7.63	7.56
C18:3	ND	ND	ND
PUFA	**7.93**	**7.63**	**7.56**
Others	0.01	0.48	0.01
IV	34	34	-

Note: PMF - palm mid-fraction, SS - shea stearin, HOSF - high-oleic sunflower oil, SFA - saturated fatty acids, MUFA - monounsaturated fatty acids, PUFA - polyunsaturated fatty acids, IV - iodine value, ND - not detected.

The TAG composition of the experimental fats is illustrated in *Table 3*. PMF consists mainly (67.6%) of the TAG species 1, 3-dipalmitoyl-2- oleoylglycerol (POP), while the majority (74.2%) of the TAG species in SS is 1, 3 distearoyl-2-oleoylglycerol (SOS) with oleic acid in the sn-2 positions. The main (66.5%) TAG species of HOSF is triolein (OOO). The three test fats consist of approximately equal oleic acid (C18:1) at the sn-2 position [(PMF = 72%, SS = 80%, HOSF = 88%)].

Postprandial Protocol

Subjects were asked to avoid consuming foods high in fat as well as not to participate in strenuous exercise the day preceding each test meal and to fast overnight beginning at 2200 hr. They were provided with a standardised low-fat meal (2–3 MJ containing, 10 g fat) to consume as their evening meal, which they were required to consume before 2200 and then to avoid eating or drinking anything except for water.

TABLE 3. TAG COMPOSITION OF THE EXPERIMENTAL FATS

TAG (% w/w)	PMF (% w/w)	SS (% w/w)	HOSF (% w/w)
PLL	0.1	ND	ND
MLP	0.2	ND	ND
OLO	0.2	ND	ND
PLO	1.0	ND	ND
PLP	8.7	ND	0.2
OOO	**0.9**	**0.3**	**66.5**
POO	3.1	0.6	9.0
POP	**67.6**	**1.0**	**0.5**
PPP	2.5	3.2	ND
SOO	0.3	7.1	5.9
POS	13.3	7.4	0.4
PPS	0.4	0.2	ND
SOS	**1.5**	**74.2**	**ND**
Others	ND	5.9	ND
SLP (°C)	31.1	37.9	<1.0

Note: TAG – triacylglycerol, PMF - palm mid-fraction, SS - shea stearin, HOSF - high-oleic sunflower oil, L - linoleic acid (18:2, n–6), M - myristic acid (14:0), O - oleic acid (18:1, n–9), P - palmitic acid (16:0), S - stearic acid (18:0), SLP - slip melting point, ND - not detected.

On the morning of the postprandial test, participants attended the Nutrition Group Clinic between 0800 and 1000 hr when a 22G" Vasofix® Brannule (Cat No. 426 8091B, B.Braun, Germany) was inserted into the antecubital vein of the forearm and held in place with a Connecta (Cat No. 394601, Becton – Dickinson, Sweden). Blood was collected by syringe and dispensed into appropriate uncapped blood collection containers. Samples for PAI-1 analysis was collected into citrate tubes while IL-6 and TNF-α analysis were collected into EDTA separator tubes and allowed to stand at room temperature for 15 min before centrifugation; plasma was then collected, aliquoted accordingly and stored frozen at -80°C until analysed.

The test meal was consumed within 10 min. Further venous blood samples were collected half-hourly until 4 hr after the test meal. Blood collection was performed by registered staff nurse supervised by medical officer or family physician using antiseptic venipuncture technique. Participants had access to water to sip as required (± 250 ml) over 4 hr. After that, the subjects were provided with an *ad-libitum* lunch.

The acute study was not carried out to 6 to 8 hr because of two main factors, namely the greatly increased subject burden involved and the necessary provision of lunch after about 4 hr may confound the results.

Analytical Methods

Haemostatic index, PAI-1 (IMUBIND, American Diagnostica, USA) and inflammatory markers IL-6 and TNF-α (R&D Systems, USA) were measured in duplicate by high-sensitivity enzyme-linked immunosorbent assay (ELISA) methods using commercial kits.

Statistical Analyses

Data were analysed by using repeated measure analysis of variance (ANOVA), using *Bonferroni post hoc* analysis by GraphPad Prism version 5 (GraphPad Software, La Jolla, CA 9203, USA) and PASW Statistics 18 to assess the significance between diets. Shapiro-Wilk's normality test was used to check for the normal distribution of data and logarithmic transformations were used when appropriate. Data are expressed as mean with 95% CI. Different superscripts attached to values in the same row across columns indicate that the values show statistically significant differences (p<0.017, Bonferroni multiple comparison test).

RESULTS

The current study shows that there are no significant differences between diets and time in plasma PAI-1 levels recorded (*Table 4*) (p=0.37). Plasma PAI-1 concentrations following PMF and SS test meals fell postprandially from the baseline until it reached 180 min - followed by an increase until the 240 min - timepoint. In addition, the SS test meal induced marginally higher (p>0.017) postprandial PAI-1 levels as compared to PMF-and HOSF-test meals.

The IL-6 (*Table 5*) and TNF-α (*Table 6*) responses did not differ significantly between the three test

TABLE 4. POSTPRANDIAL CHANGES IN PAI-1 FOLLOWING THE THREE TEST MEALS

Time (min)	HOSF	SS	PMF
0	28.99 (23.49, 34.49)	29.12 (23.73, 34.50)	27.83 (22.71, 32.95)
60	22.39 (18.34, 26.44)	25.00 (20.67, 29.34)	24.33 (20.45, 28.21)
120	20.33 (16.45, 24.20)	23.94 (19.17, 28.72)	25.30 (20.95, 29.65)
180	21.43 (17.58, 25.29)	22.46 (17.80, 27.13)	20.21 (16.47, 23.96)
240	26.04 (20.69, 31.40)	25.70 (20.31, 31.09)	23.78 (19.07, 28.48)

Note: PAI-1 - plasminogen activator inhibitor-1. Values are geometric means; 95% CI in parentheses; N = 36 (18 men, 18 women) for all treatments. Values were log-transformed, analysed by repeated-measures ANOVA, and showed a diet × time interaction (p=0.000); the meal × gender, time × gender and meal × gender × time interactions were not significant.

meals given. However, the SS-test meal induced marginally higher postprandial IL-6 but lower TNF-α concentrations (p>0.017) compared to the PMF- and HOSF-test meals.

DISCUSSION

Postprandial inflammation affects the haemostatic balance and the vascular system, and inflammatory responses were reported to peak 4 to 8 hr after a meal (Margioris, 2009). To date, data on postprandial haemostatic and inflammatory responses induced by different dietary fats lack a consensus. As such, we conducted the present study to compare the acute effects of palmitic acid-rich PMF (POP-type fat), stearic acid-rich SS (SOS-type fat) and oleic acid-rich HOSF (OOO-type fat) on the haemostatic marker, PAI-1 and the inflammatory markers, IL-6 and TNF-α.

The PAI-1 is a key regulator of fibrinolysis and increased levels of this haemostatic marker have been associated with an increased risk of CVD (Brazionis,

TABLE 5. POSTPRANDIAL RESPONSES IN IL-6 FOLLOWING THE THREE TEST MEALS

Time (min)	HOSF	SS	PMF
0	10.61 (1.17, 20.05)	13.96 (-2.61, 30.53)	9.05 (0.72, 17.39)
60	9.21 (1.17, 17.26)	9.98 (-0.62, 20.57)	8.85 (-0.04, 17.75)
120	9.89 (1.34, 18.45)	10.18 (-0.14, 20.51)	8.53 (0.04, 17.02)
180	10.00 (0.64, 19.36)	10.16 (-0.17, 20.50)	8.61 (0.34, 16.88)
240	10.02 (1.70, 18.34)	10.70 (0.55, 20.85)	7.12 (-0.39, 14.64)

Note: IL-6 interleukin-6. Values are means; 95% CI in parentheses; N = 36 (18 men, 18 women) for all treatments. Values were analysed by repeated-measures ANOVA, and diet × time, the meal × gender, time × gender and meal × gender × time interactions were not significant (p>0.017).

TABLE 6. POSTPRANDIAL RESPONSES IN TNF-α FOLLOWING THE THREE TEST MEALS

Time (min)	HOSF	SS	PMF
0	68.53 (-18.49, 155.56)	50.10 (-12.66, 112.87)	81.25 (-22.93, 185.43)
60	54.10 (-14.50, 122.71)	47.25 (-13.39, 107.89)	80.83 (-23.97, 185.62)
120	62.67 (-20.89, 146.23)	49.08 (-14.27, 112.43)	80.87 (-24.47, 186.21)
180	61.76 (-21.29, 144.81)	49.94 (-15.68, 115.57)	80.79 (-24.69, 186.26)
240	66.90 (-20.29, 154.09)	51.01 (-15.49, 117.51)	80.78 (-24.58, 186.15)

Note: TNF-α - tumour necrosis factor-alpha. Values are geometric means; 95% CI in parentheses; (n = 36; 18 men, 18 women) for all treatments. Values were log-transformed, analysed by repeated-measures ANOVA, and diet × time, the meal × gender, time × gender and meal × gender × time interactions were not significant (p>0.017).

2008; De Taeye *et al*., 2005). In the present study, there were no significant differences obtained between test meals and time for plasma PAI-1 levels (p=0.37). However, postprandial plasma PAI-1 concentrations following PMF-, SS-, and HOSF-test meals fell some 27.4%, 22.9% and 26.1%, respectively from the baseline by the 180 min-timepoint. From the 180 min- to the 240 min-timepoint, plasma PAI-1 levels across all three groups recovered somewhat, reducing the initial 180 min drop from baseline by approximately half. In addition, SS (SOS)-fat induced a marginally higher (p>0.017) postprandial PAI-1 level as compared to PMF and HOSF.

The current findings differ somewhat from that reported by Sanders *et al*. (2001) whereby cocoa butter (SOS-type of fat) induced lower postprandial PAI-1 concentrations as compared to an oleate-rich (OOO-type of fat), but was similar in that there was no significant differences between the test meals or significant diet × time interaction. Our findings also differed from that of Delgado-Lista *et al.* (2008) which showed adverse effects for SFA-rich meal (*i.e.* raising postprandial PAI-1 levels) versus a monounsaturated fatty acid (MUFA)-rich meal.

Elevated PAI-1 levels have been shown to be associated with elevated fasting plasma TAG concentrations, obesity and insulin resistance syndrome (Duttaroy, 2005; De Taeye *et al*., 2005). Our study demonstrated that SS (SOS)-fat induced a marginally higher postprandial PAI-1 concentration as compared with the PMF (POP) and HOSF (OOO) fats. Our findings therefore suggest that postprandial lipemia induced by test fats rich in palmitic or oleic acid at the sn-1 and sn-3 positions does not impair fibrinolytic activity. The marginal difference of PAI-1 levels between the test meals might be due to a difference in insulin sensitivity levels and which then affects postprandial insulin secretion (Delgado-Lista *et al*., 2008).

Cytokines IL-6 and TNF-α are key mediators in inflammation and produced by immune cells, monocytes, macrophages, endothelial cells as well as in vascular smooth muscle cells (Galli and Calder, 2009). The TNF-α plays a major role in the cytokine cascade as it stimulates the synthesis of other cytokines (Lind, 2003) while IL-6 is a mediator of the acute-phase response and the primary determinant of CRP production (Heinrich, 1990). These cytokine markers of inflammation are strong predictors of cardiovascular disease (Blake and Ridker, 2002) as it has been reported that elevated level of IL-6 and TNF-α are related to insulin resistance and obesity incidences (Kern *et al*., 2001; Pradhan *et al*., 2001).

The IL-6 and TNF-α responses did not differ significantly between the three test meals given. These results are in agreement with that reported by Tholstrup *et al*. (2011) which showed no significant difference in mean postprandial IL-6 values after healthy adults were given a cocoa butter-enriched or olive oil-enriched meals. However, Baer *et al*. (2004) reported that IL-6 concentrations were lower after the consumption of an oleic acid-rich diet compared to stearic acid-rich and palmitic acid-rich diets. This general lack of agreement between our study and that of Baer *et al*. (2004) could be due to 'anthropomorphic' differences of subjects, including age, recruited for different studies.

CONCLUSION

The different stereospecificity and the predominant type of SFA in the three test fats – palm mid-fraction (PMF; POP- rich), shea stearin (SS; SOS-rich) and high-oleic sunflower oil (HOSF; OOO-rich) induced similar postprandial responses in the haemostatic variable, PAI-1 and the inflammatory markers IL-6 and TNF-α in the present acute study.

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