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High-Fat Diets Rich in N-3 Polyunsaturated Fatty Acids Delay Onset of Insulin Resistance in Rats

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Abstract: Insulin resistance is a growing worldwide syndrome that predispose human to a number of chronic diseases such as diabetes and cardiovascular diseases. Various studies have indicated that insulin action is highly influenced by diet compositions particularly dietary fat intake and proportion of n-3 and n-6 Polyunsaturated Fatty Acids (PUFA). The current study was designed to assess the influence of High Fat Diet (HFD) with different n-6: n-3 Fatty Acid Ratios (FAR) on insulin sensitivity, plasma triacylglycerol (TAG) and lipoprotein profile. Forty male *Sprague-Dawley* rats were randomly allocated into four groups and received the isocaloric high fat diets enriched with either high n-6: n-3 fatty acid ratio (HFAR), medium n-6: n-3 fatty acid ratio (MFAR), low n-6: n-3 fatty acid ratio (LFAR) and compared with control rats fed standard chow (CTRL). The plasma insulin level of HFAR fed rats manifested significantly ($P<0.05$) higher concentration in most of the time points compared to the other groups. The corresponding insulin AUC (ng/L/120min) and insulin sensitivity index of HFAR fed rats appeared to be significantly higher ($P<0.05$) than CTRL (335.5±38.5), LFAR (273.7±37.6) and MFAR (265.9±21.7) groups. Blood lipid profile were found to be healthier in the LFAR and MFAR supplemented groups with significantly ($P<0.05$) lower total cholesterol and TAG levels. This study showed the possible protective effect against insulin resistance when low n-6: n-3 fatty acid ratio in high fat diets are applied in a rat model.

Key words: n-6: n-3 fatty acid ratios, insulin sensitivity, triacylglycerol, total cholesterol, insulin, rat, chronic diseases

INTRODUCTION

Insulin resistance is a central component of type 2 diabetes mellitus, a chronic disease with multiple metabolic disorders (Trout *et al*., 2007). Studies involving animals and human suggest the role of macro nutrients composition of diet as important environmental determinants of insulin resistance (Storlien, 2000; Vessby, 2000; Riccardi *et al*., 2004; Lara-Castro and Garvey, 2004). The influence of diet on insulin sensitivity is mediated by its energy content and nutrient composition, in particular by different types of dietary fatty acids (Riccardi *et al*., 2004). High intakes of Saturated Fatty Acids (SFA) lead to insulin resistance, whereas (n-3) PUFA prevent the development of insulin resistance (Ibrahim *et al*., 2005; Lombardo and Chicco, 2006). High fat diet based on n-6 fatty acid has been shown to reduce insulin sensitivity in rat as well (Storlien *et al*., 2000). Moreover, it has been mentioned that the current dietary intake characterized by elevated n-6: n-3 fatty acid ratio considered having an adverse effect on the action of insulin (Dubnov and Berry, 2004). Thus, establishment of optimal dietary intake of n-3: n-6 ratio is crucial for the maximum health benefits of PUFA. Besides to their influence on plasma glucose and insulin concentration, dietary fatty acid composition modulates plasma lipid levels (Hong *et al*., 2003). Supplementary to measurement of glucose and insulin concentration, assessment of plasma TAG and lipoprotein cholesterol profile serve as an important biomarker of insulin resistance (Hannon *et al*., 2006). High levels of plasma TAG and Low-density Lipoprotein Cholesterol (LDL-C) plus lower level of High-density Lipoprotein Cholesterol (HDL-C) are regarded as dyslipidemia associated with insulin resistance (Bonora *et al*., 1998; Howard, 2004). Although the TAG lowering effect of long chain n-3 fatty acids have been reported consistently, its effect on LDL-C and HDL-C still has remained controversial (Hatahet *et al*., 2003; Mohamed *et al*., 2002; Madden *et al*., 2008). It has been also stated that Saturated Fatty Acids (SFA) produce an

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increase in TAG, total serum cholesterol and mainly in the LDL-C concentration (Karmally, 2005). However, unlike the effect of n-3 fatty acids, the hypolipidemic effect of n-6 fatty acid is not consistently reported. Jeffery *et al.* (1996) stated that the plasma concentration of TAG decreased progressively with the decrease in the n-6: n-3 ratio of the diet. Thus an objective of the present study was to assess insulin sensitivity response, plasma lipoprotein cholesterol and TAG concentrations in response to dietary intake of high fat diet enriched with various n-6: n-3 FAR in *Sprague-Dawley* rats.

MATERIALS AND METHODS

Animals, diets and experimental design: Forty male *Sprague-Dawley* rats (9 weeks old) were obtained from an animal resource unit of University Putra Malaysia and used in this study. They were placed individually in polycarbonate cages with shaved wood beddings. Rats were maintained in a well-ventilated room environment with a temperature of 23±1°C and 12 hour light-dark cycle. After one week of acclimatization with standard chow diet (Ridley Agriproducts, Sydney, Australia) (Table 1), rats were randomly assigned into four experimental groups: (1) Rats fed standard chow diet as a control group (CTRL) and the other three treatment groups received standard chow diet enriched with: (2) 6.67% w/w menhaden oil +3.3% w/w soya bean oil (LFAR): (3) 3.3% w/w menhaden oil+6.67% w/w soya bean oil (MFAR): (4) 10% w/w butter (HFAR). Rats in all groups received experimental diets for 22 weeks. All experimental protocols were in accordance with the guidelines of the Institute of Animal Care and Use Committee, Faculty of Veterinary Medicine, Universiti Putra Malaysia.

Measurement of insulin sensitivity: As a measure of insulin sensitivity, Intraperitoneal Glucose Tolerance Test (IPGTT) and Intraperitoneal Insulin Tolerance Test (IPITT) were performed on day 0 and 20th week of dietary intervention. The corresponding area under the curves of IPGTT and IPITT were also determined using trapezoidal rule (Ibrahim *et al.*, 2005). In addition, insulin sensitivity index of baseline and 20th week tests were determined as a product of glucose and insulin AUC (Hsu *et al.*, 2007). This index serves as an indirect mean to assess *in vivo* insulin action (Subramanian *et al.*, 2008).

Intraperitoneal Glucose Tolerance Test (IPGTT): An Intraperitoneal glucose tolerance test was performed using the method described by Scarpace *et al.* (2005). It was performed on ten randomly selected rats at day 0 and then on all rats at the 20th week of dietary treatment. The IPGTT procedures were carried out on overnight-fasted rats. Blood glucose was measured using a glucometer (Accu-Check, Roche Diagnostics Corporation, USA) immediately before and at 15, 45, 90 and 120 min after intraperitoneal glucose (2g/kg, Sigma-Aldrich, St. Louis, MO, USA) administration.

Table 1: Chemical constituents of laboratory chow diet (dry-weight basis)

Constituent (per 100 g feed)	Diet (%)
Crude fat (g)	3.0
Crude protein (g)	20.0
Carbohydrate (g)	51.0
Crude fiber (g)	7.3
Ash (g)	9.2
Calcium	0.9
Phosphorus	0.5

Intraperitoneal Insulin Tolerance Test (IPITT): Similar to the IPGTT, IPITT was also carried out in similar periods in accordance with the procedure of Ropelle *et al.* (2006). Rats were fasted overnight and then were infused with human recombinant insulin (Humulin, Eli Lilly and Company Indianapolis, USA) intraperitoneally at a dose rate of 1.5 IU/kg body weight. Blood glucose was then measured immediately before and at 15, 45, 90 and 120 min after insulin injection.

Plasma insulin quantification: Plasma insulin was measured using blood samples collected immediately before and 15, 45, 90 and 120 minutes after intraperitoneal glucose load during IPGTT. Blood was collected from the tail tip into EDTA coated blood tubes and centrifuged at 1500xg for 10 min. Plasma insulin concentrations were measured using a rat insulin ELISA kit (Linco Research, St Charles, MO, USA) according to the manufacturer's protocol.

Blood sampling and the preparation of blood samples: At the end of week 22 of dietary intervention, rats were fasted overnight. Blood from abdominal aorta was collected under anesthesia using an EDTA-coated vacutainer tube. After blood sampling, rats were sacrificed by decapitation. Collected blood samples were centrifuged at 3000xg for 10 min at 4°C and then the separated plasma was utilized for fatty acid analysis as well as determination of lipoprotein and triacylglycerol concentration. The remaining Red Blood Cell (RBC) samples undergo hemolysis by using hypotonic phosphate buffer saline for preparation of RBC membrane. In addition, a portion of gluteal muscle which represents large muscle mass was also collected and kept at -80°C for fatty acid analysis.

Lipid extraction and fatty acid methyl ester preparation: Total lipid extraction from the treatment diets, plasma, skeletal muscle and red blood cell membrane were carried out in accordance to the

method of Folch *et al.* (1957), modified by Rajion *et al.* (1985) as described by Ebrahimi *et al.* (2013). Transmethylation of the extracted fatty acids to Fatty Acid Methyl Esters (FAME) were carried out using 14% methanolic-boron trifluoride following methods of (Association of official analytical chemists) AOAC (1990). A known concentration of heneicosanoic acid (21:0) (Sigma Chemicals CO., St. Louis, Missouri, USA) was added to each sample before the start of transmethylation as an internal standard for quantification of individual fatty acid concentration within the sample.

Gas liquid chromatography: Fatty acid methyl esters were analyzed by Hewlett-Packard Gas-Liquid Chromatography (Hewlett Packard Avondale, PA, USA) using Supelco SP-2330 (30 m, 0.25 mm ID, 0.20 μm film thickness) column (Supelco, Inc Bellefonte, PA, USA). Ultra pure nitrogen obtained from the nitrogen generator (Dominick Hunter, Leicester, UK) was used as the carrier gas at 40 mL/min. Ultra pure hydrogen (Dominick Hunter, Leicester, UK) and compressed air were employed for flame ionization detector in a gas liquid chromatography. The injector and detector temperatures were set at 250°C. The column temperature was set at a range of 100-190°C with temperature programming at an incremental rate of 7.2°C/min to facilitate optimal separation. The identification of individual fatty acids was made by comparison of retention time with the peaks of authentic standards based on their equivalent chain length number. Peak areas were determined using HP-3393A Integrator (Hewlett Packard Avondale, PA, USA).

Plasma lipoprotein determination: Total cholesterol, LDL-C, HDL-C and triacylglycerol concentration were determined by Hitachi 902 automatic chemistry analyzer using their respective kits (Roche Diagnostics, Basel, Switzerland).

Data analysis: Changes in the glucose and insulin level along the time point and treatment groups were compared across all treatment groups using blocked-time effect two ways ANOVA during IPGTT, IPITT and periodic insulin concentration measurements. Insulin and glucose AUC as well as plasma lipid data were compared across treatment groups using a simple ANOVA procedure. *Post hoc* comparisons were performed using Least Significant Difference (LSD) to elucidate the significant difference in means. All values were presented as means±standard error and significance was considered at P<0.05.

RESULTS

Intraperitoneal glucose tolerance test (IPGTT): The response of rats to the performed IPGTT at day 0 and 20th week of dietary intervention and the corresponding area under the curve were presented below (Fig. 1-2).

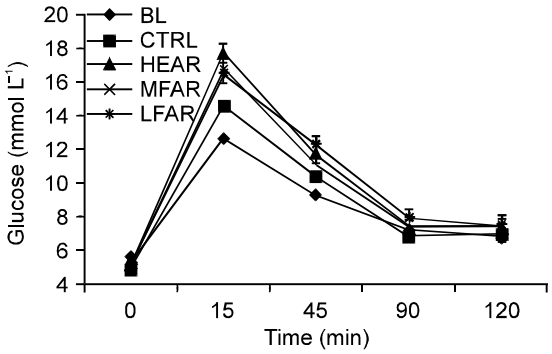

Fig. 1: Baseline and 20th week IPGTT curve of rats. There was no significant difference (P>0.05) between different groups at the same time points. BL: Baseline, CTRL: Control group, HFAR: High n-6: n-3 fatty acid ratio group, MFAR: Medium n-6: n-3 fatty acid ratio group, LFAR: Low n-6: n-3 fatty acid ratio group

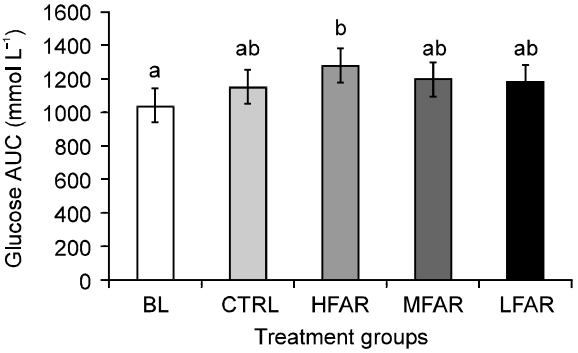

Fig. 2: Glucose AUC of baseline and 20th week IPGTT of rats. Different letters among groups indicate significant differences (P<0.05). BL: Baseline, CTRL: Control group, HFAR: High n-6: n-3 fatty acid ratio group, MFAR: Medium n-6: n-3 fatty acid ratio group, LFAR: Low n-6: n-3 fatty acid ratio group

The IPGTT test performed on day zero and the 20th week showed a similar trend of curve with blood glucose reaching the peaked at time point 15 minutes after glucose administration. Blood glucose decreased gradually after the 15 minute time point and stabilized at about 90 minutes. Overall, the glucose reading for LFAR and MFAR group which was supplemented with n-3 and n-6 PUFA was significantly (P<0.05) lower when compared to HFAR and CTRL group at most of the time points. The glucose AUC for the 20th week of IPGTT revealed a similar result for all the treatment groups.

Intraperitoneal insulin tolerance test (IPITT): The result from IPITT showed an inverse picture of the IPGTT curve (Fig. 3-4). The glucose level decreased gradually until 45

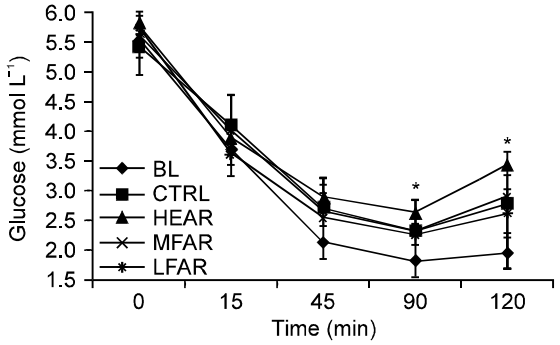

Fig. 3: Baseline and 20th week IPITT curve of rats. *: shows significant difference (P<0.05) at the same time point in HFAR and other groups. BL: Baseline, CTRL: Control group, HFAR: High n-6: n-3 fatty acid ratio group, MFAR: Medium n-6: n-3 fatty acid ratio group, LFAR: Low n-6: n-3 fatty acid ratio group

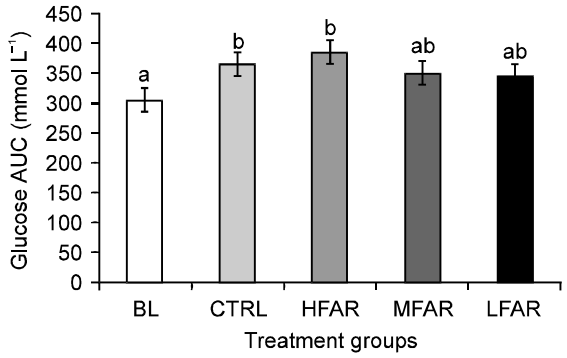

Fig. 4: Glucose AUC of baseline and 20th week IPITT of rats. Different letters among groups indicate significant differences (P<0.05). BL: Baseline, CTRL: Control group, HFAR: High n-6: n-3 fatty acid ratio group, MFAR: Medium n-6: n-3 fatty acid ratio group, LFAR: Low n-6: n-3 fatty acid ratio group

minutes after insulin injection and stabilized to normal level after the 90th minute. Overall, the HFAR group exhibited significant (P<0.05) higher blood glucose concentration from 90 minutes time point when compared with other treatment groups and baseline. The total glucose concentration with time as depicted by the AUC values showed the LFAR and MFAR group had a better control of blood glucose in the presence of insulin when compared to HFAR and CTRL group.

Plasma insulin and insulin sensitivity index: The plasma insulin curve and the corresponding AUC were calculated and shown in the Figures below (Fig. 5-6). The insulin concentration curves for all the treatment

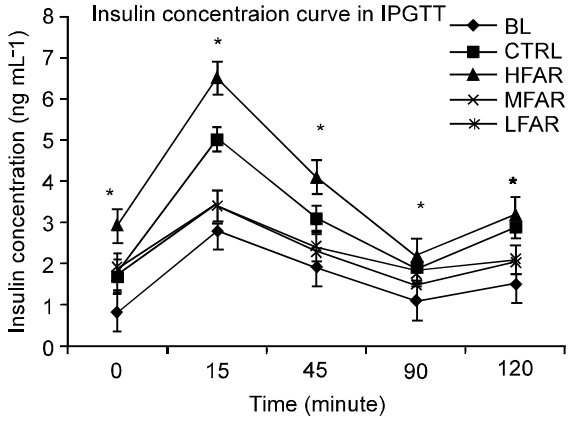

Fig. 5: Insulin concentration curve of baseline and 20th week IPGTT of rats. *: shows significant difference (P<0.05) at the same time point in HFAR and other groups. BL: Baseline, CTRL: Control group, HFAR: High n-6: n-3 fatty acid ratio group, MFAR: Medium n-6: n-3 fatty acid ratio group, LFAR: Low n-6: n-3 fatty acid ratio group

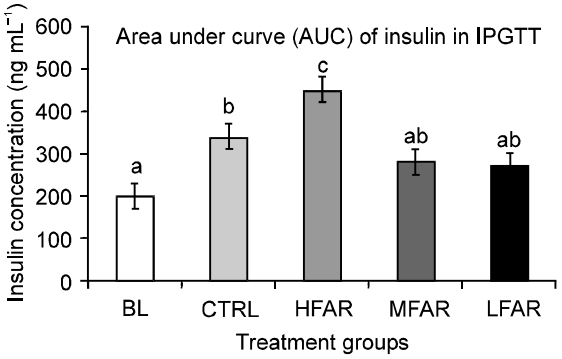

Fig. 6: Insulin AUC of baseline and 20th week IPGTT of rats. Different letters among groups indicate significant differences (P<0.05). BL: Baseline, CTRL: Control group, HFAR: High n-6: n-3 fatty acid ratio group, MFAR: Medium n-6: n-3 fatty acid ratio group, LFAR: Low n-6: n-3 fatty acid ratio group

groups showed a similar trend where the insulin concentration in the blood reaches its peak at the 15th minute. This was due to insulin being released into blood to regulate the blood glucose level. The concentration of insulin gradually decreases as the time increases due to its function in regulating glucose uptake. As seen from the insulin concentration curve, HFAR and CTRL group showed a wide departure from the baseline insulin level after 20 weeks of dietary intervention. At the 20th week, the HFAR group exhibited significantly higher (P<0.05) insulin concentration at all time points. For the LFAR and MFAR which was

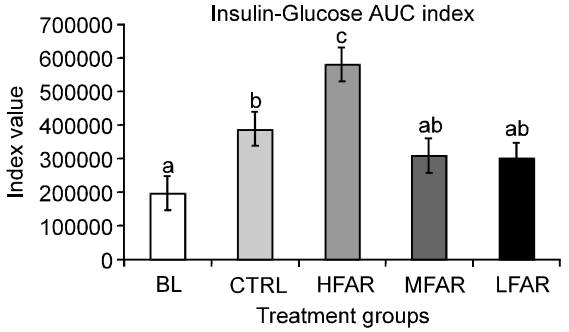

Fig. 7: Insulin-glucose AUC index at day 0 and after 20th week. Different letters among groups indicate significant differences (P<0.05). BL: Baseline, CTRL: Control group, HFAR: High n-6: n-3 fatty acid ratio group, MFAR: Medium n-6: n-3 fatty acid ratio group, LFAR: Low n-6: n-3 fatty acid ratio group

supplemented with n-3 and n-6 PUFA showed a more moderate insulin secretion and regulation.

The trend of the insulin concentration was also clearly noted on the AUC graph shown. HFAR group showed significant (P<0.05) high values of insulin concentration after intraperitoneal glucose administration when compared to CTRL, LFAR and MFAR groups. The glucose-insulin index (Fig. 7) revealed significantly higher value (P<0.05) for the HFAR rats. The index was used to measure the level of occurrence of insulin resistance. In addition, the glucose insulin index for LFAR and MFAR group which was supplemented with n-3 and n-6 PUFA did not exhibit significant variation in terms of their index when compared to the baseline rats.

Dietary fatty acid profile: The fatty acid profile of experimental diets was presented in Table 2. The results showed that HFAR diet contained significantly highest (P<0.05) saturated fatty acid amounting for more than 50% of the total fatty acids. On the other hand, LFAR diet exhibited highest amount of n-3 fatty acid (39.2%) doubling the concentration detected in MFAR diet (16.1%). However, in CTRL and HFAR treatment diets n-3 fatty acid accounted for less than 1% of the total fatty acid. In terms of the total n-6 fatty acid, MFAR diet took the lead comprising 39.2%, followed by 26.4% in LFAR diet. The fatty acid composition in tissues such as plasma, red blood cell membrane and skeletal muscle were presented in Table 3-5 strongly reflects the fatty acid composition in diet supplemented.

Plasma fatty acid profile: The fatty acid composition in plasma of animals in each experimental group was shown in Table 3. In general, the plasma fatty acids maintained a similar unsaturated to saturated fatty acid composition regardless of the dietary fatty acid

Table 2: Fatty acid profile of different experimental diets

	Experimental diets				
Fatty acid (g/100g)	HFAR	LFAR	MFAR	CTRL	SEM
Caproic acid (6:0)	0.8	n/d	n/d	n/d	0.20
Caprylic acid (8:0)	4.2	n/d	n/d	n/d	1.05
Capric acid (10:0)	0.9	n/d	n/d	n/d	0.23
Lauric acid (12:0)	7.5^{b}	0.13^{a}	n/d	n/d	1.86
Myristic acid (14:0)	4.6^{c}	4.8^{c}	2.5^{b}	0.6^{a}	0.99
PA (15:0)	n/d	0.43^{c}	0.23^{a}	n/d	0.10
Palmitic acid (16:0)	27.6^{c}	17.8^{b}	15.0^{b}	16.7^{a}	2.83
Palmitoleic acid (16:1)	n/d	5.2^{b}	2.7^{a}	n/d	1.25
HA (17:0)	n/d	n/d	0.33	n/d	0.08
Stearic acid (18:0)	5.9^{c}	4.4^{b}	4.5^{b}	5.7^{a}	0.39
Oleic acid (18:1)	25.1^{d}	18.4^{b}	21.8^{c}	23.4^{a}	1.43
LA (18:2 (n-6))	19.5^{b}	25.8^{b}	38.9^{c}	48.1^{a}	2.42
LA (18:3 (n-3))	0.33^{b}	0.44^{c}	0.6^{c}	0.31^{a}	0.07
Arachidic acid (20:0)	1.8^{a}	3.6^{b}	5	4.6^{a}	0.71
EA (Cis-11- 20:1)	0.39^{a}	1^{b}	n/d	n/d	0.24
AA (20:4 (n-6)	n/d	0.64^{a}	0.2^{b}	n/d	0.15
EA (20:5 (n-3))	n/d	8.8^{b}	4.5^{a}	n/d	2.11
Behenic acid (22:0)	0.4^{b}	n/d	n/d	0.57^{a}	0.14
Erucic acid (22:1)	n/d	0.53^{b}	0.2^{a}	n/d	0.13
DA (22:5 (n-3))	n/d	1.2^{b}	0.6^{a}	n/d	0.29
DA (22:6 (n-3))	n/d	5.6^{b}	2.5^{a}	n/d	1.33
Lignoceric acid (24:0)	n/d	0.48	n/d	n/d	0.12
TSFA	54.1^{c}	32.1^{b}	27.4^{b}	28.3^{a}	6.29
TUFA	45.9^{b}	67.8^{c}	72.4^{d}	71.7^{a}	6.27
TMFA	26.1^{c}	25.2^{b}	25.1^{b}	23.4^{a}	0.55
Total PUFA n-3	0.33^{a}	16.1^{c}	8.2^{b}	0.31^{a}	3.77
Total PUFA n-6	19.5^{b}	26.4^{c}	39.2^{d}	48.1^{a}	6.38
n-6:n-3 ratio	59.8^{b}	1.6^{a}	4.8^{a}	154.8^{c}	35.76
U:S ratio	0.85^{a}	2.1^{b}	2.6^{c}	2.5^{c}	0.41
P:S ratio	0.37^{a}	1.3^{b}	1.7^{c}	1.7^{c}	0.32

^{a,b,c,d}Values with different superscripts within rows are significantly different (P<0.05), n/d: not detected, S: Saturated, U: Unsaturated, P: Polyunsaturated. SEM: Standard error of means. BL: Baseline, CTRL: Control group, HFAR: High n-6: n-3 fatty acid ratio group, MFAR: Medium n-6: n-3 fatty acid ratio group, LFAR: Low n-6: n-3 fatty acid ratio group, PA: Pentadecanoic acid, HA: Heptadecanoic acid, LA: Linolenic acid, EA: Eicosenoic acid, AA: Arachidonic acid, EA: Ecosapentanoic acid, DA: Docosapentanoic acid, DA: Docosahexanoic acid, TSFA: Total saturated fatty acids, TUFA: Total unsaturated fatty acids, TMFA: Total monounsaturated fatty acids

supplemented. All groups had around two thirds of their plasma fatty acid as unsaturated fatty acids but the levels of individual unsaturated fatty acids clearly reflect the type of fatty acid supplemented. In the LFAR group, the plasma n-3 PUFA was the highest (P<0.05) at 12.8%. This clearly reflects the higher ratio of n-3 fatty acids supplemented from menhaden oil in the diet. The plasma from MFAR group had the highest n-6 PUFA with the majority of n-6 PUFA dominated by the linoleic acid. It takes up 23.8% of the total 53.7% of n-6 PUFA contributed in the plasma. Both HFAR and CTRL rats had negligible total n-3 fatty acids but high amounts of n-6 fatty acids, a fact contributed by Arachidonic Acid (AA).

Red blood cell membrane fatty acid profile: The red blood cell membrane fatty acid composition of animals in experimental groups was shown in Table 4. MFAR group showed significantly (P<0.05) higher n-6 fatty acid of 34.6% in red blood cell membrane compared to CTRL

Table 3: Plasma fatty acid profile of treatment groups

	Experimental diets				
Fatty acid (g/100ml)	HFAR	LFAR	MFAR	CTRL	SEM
Capric acid (10:0)	n/d	0.5^{a}	0.5^{a}	n/d	0.14
Lauric acid (12:0)	0.3^{a}	0.3^{a}	0.2^{a}	n/d	0.07
Myristic acid (14:0)	0.7^{ab}	1.0^{c}	0.7^{bc}	0.5^{a}	0.10
Pentadecanoic acid (15:0)	0.5^{a}	0.8^{b}	0.7^{ab}	0.6^{ab}	0.06
Palmitic acid (16:0)	19.9^{b}	19.0^{ab}	16.6^{a}	18.1^{ab}	0.70
Palmitoleic acid (16:1)	0.5^{a}	1.2^{b}	1.2^{b}	0.7^{a}	0.18
Heptadecanoic acid (17:0)	0.5^{a}	0.9^{b}	0.7^{ab}	0.6^{a}	0.09
Stearic acid (18:0)	12.4^{b}	10.4^{a}	11.2^{a}	11.7^{ab}	0.42
Oleic acid (18:1)	12.3^{a}	11.2^{a}	13.2^{a}	9.9^{a}	0.71
Linoleic acid (18:2 (n-6))	19.3^{a}	24.3^{a}	23.8^{a}	22.3^{a}	1.13
Linolenic acid (18:3 (n-3))	n/d	0.01	n/d	0.4	0.01
Arachidic acid (20:0)	0.4^{a}	0.9^{b}	0.7^{b}	0.8^{b}	0.11
Cis-11-Eicosenoic (20:1)	0.6^{ab}	0.8^{b}	0.4^{a}	0.4^{a}	0.10
Cis-11, 14, 17-Eicosatrienoic (20:3)	0.5^{ab}	n/d	0.7^{b}	0.5^{a}	0.15
Arachidonic acid (20:4 (n-6))	28.9^{b}	14.6^{a}	20.1^{a}	30.9^{b}	1.81
Ecosapentanoic acid (20:5 (n-3))	n/d	5.4^{b}	2.5^{a}	n/d	1.28
Behenic acid (22:0)	1.5^{b}	1.5^{ab}	1.6^{ab}	0.8^{a}	0.18
Docosapentanoic acid (22:5 (n-3))	n/d	1.1^{b}	0.6^{a}	n/d	0.27
Docosahexanoic acid (22:6 (n-3))	1.8^{a}	6.4^{b}	4.6^{b}	2.0^{a}	0.10
Total saturated fatty acids^{ns}	36.2	35.3	33.0	33.0	0.82
Total unsaturated fatty acids^{ns}	63.8	64.7	67.0	67.0	0.82
Total monounsaturated fatty acids^{ns}	13.4	13.2	14.7	11.0	0.79
Total PUFA n-3	1.8^{a}	12.91^{c}	7.7^{b}	2.4^{a}	0.60
Total PUFA n-6	48.7^{b}	38.9^{a}	43.9^{ab}	53.2^{b}	3.05
n-6: n-3ratio	34.1^{b}	3.02^{a}	5.7^{a}	22.17^{b}	1.91
U:S ratio^{ns}	1.8	1.8	2.04	2.0	0.07
P:S ratio	1.4^{a}	1.5^{ab}	1.6^{bc}	1.7^{c}	0.06

^{a,b,c}Values with different superscripts within rows are significantly different (P<0.05), n/d: not detected, S: Saturated, U: Unsaturated, P: Polyunsaturated, ns: not significant. SEM: Standard error of means. BL: Baseline, CTRL: Control group, HFAR: High n-6: n-3 fatty acid ratio group, MFAR: Medium n-6: n-3 fatty acid ratio group, LFAR: Low n-6: n-3 fatty acid ratio group

Table 4: Fatty acid composition of RBC membrane of rats

	Experimental diets				
Fatty acid (g/100mL)	HFAR	LFAR	MFAR	CTRL	SEM
Myristic acid (14:0)^{ns}	0.9	1.0	0.7	1.0	0.07
Pentadecanoic acid (15:0)^{ns}	0.7	1.0	0.7	1.0	0.09
Palmitic acid (16:0)	27.6^{b}	24.1^{ab}	23.7^{ab}	27.8^{a}	1.10
Palmitoleic acid (16:1)^{ns}	1.2	1.4	1.2	2.0	0.19
Heptadecanoic acid (17:0)	0.6^{a}	1.1^{c}	0.8^{bc}	1.0^{ab}	0.11
Stearic acid (18:0)	19.1^{b}	16.1^{ab}	14.2^{ab}	18.1^{a}	1.09
Oleic acid (18:1)^{ns}	9.0^{a}	8.3^{a}	9.3^{a}	10.9^{a}	0.55
Linoleic acid (18:2 (n-6))	9.5^{b}	14.4^{b}	13.3^{c}	8.9^{a}	1.37
Linolenic acid (18:3 (n-3))	n/d	n/d	0.3^{b}	0.3^{a}	0.001
Cis-11, 14-Eicosadieonic (20:2)	n/d	n/d	0.5^{b}	0.5^{a}	0.001
Cis-11, 14, 17-Eicosatrienoic (20:3)	0.4^{a}	n/d	0.8^{b}	0.8^{a}	0.12
Arachidonic acid (20:4 (n-6))	22.4^{b}	15.7^{a}	20.8^{b}	21.9^{a}	1.54
Ecosapentanoic acid (20:5 (n-3))	n/d	3.4^{b}	1.6^{a}	n/d	0.14
Behenic acid (22:0)	4.5^{a}	9.6^{b}	5.3^{ab}	3.4^{a}	1.36
Docosapentanoic acid (22:5 (n-3))	1.2^{a}	2.6^{b}	2.1^{b}	0.5^{a}	0.17
Docosahexanoic acid (22:6 (n-3))	1^{a}	4.6^{b}	3.9^{b}	0.8^{a}	0.98
Lignoceric acid (24:0)	2.0^{b}	n/d	0.7^{a}	1.7^{a}	0.34
Total saturated fatty acids	53.4^{b}	52.9^{ab}	45.4^{ab}	52.3^{a}	2.02
Total unsaturated fatty acids	46.6^{ab}	47.1^{a}	54.6^{b}	47.7^{a}	1.98
Total monounsaturated fatty acids^{ns}	10.2^{a}	9.7^{a}	10.5^{a}	12.9^{a}	0.71
Total PUFA n-3	2.2^{a}	10.5^{b}	7.9^{b}	1.6^{a}	0.17
Total PUFA n-6	31.9^{ab}	30.1^{a}	34.1^{b}	32.8^{a}	1.57
n-6 : n-3 ratio	14.50^{b}	2.8^{a}	4.3^{a}	19.25^{c}	1.28
U:S ratio	0.8^{a}	0.9^{a}	1.2^{b}	0.91^{a}	0.09
P:S ratio	0.7^{ab}	0.7^{ab}	0.9^{b}	0.62^{a}	0.06

^{a,b,c}Values with different superscripts within rows are significantly different (P<0.05), n/d: not detected, S: Saturated, U: Unsaturated, P: Polyunsaturated, ns: not significant. SEM: Standard error of means. BL: Baseline, CTRL: Control group, HFAR: High n-6: n-3 fatty acid ratio group, MFAR: Medium n-6: n-3 fatty acid ratio group, LFAR: Low n-6: n-3 fatty acid ratio group

Table 5: Muscle tissue fatty acid profile of treatment groups

	Experimental diets				
Fatty acid (mg/100g)	HFAR	LFAR	MFAR	CTRL	SEM
Lauric acid (12:0)	2.3	n/d	n/d	n/d	0.58
Myristic acid (14:0)	2.2^{b}	2.1^{b}	1.1^{a}	0.58^{a}	0.39
Pentadecanoic acid (15:0)	0.3^{a}	0.42^{b}	0.32^{a}	0.35^{a}	0.03
Palmitic acid (16:0)	32.5^{c}	18.2^{b}	16.3^{a}	20.3^{a}	1.65
Palmitoleic acid (16:1)	0.6^{a}	3.1^{b}	1.5^{a}	1.6^{a}	0.50
Heptadecanoic acid (17:0)	0.3^{a}	0.59^{b}	0.47^{a}	0.51^{a}	0.06
Stearic acid (18:0)	9.5^{b}	5.9^{ab}	6.4^{a}	10.1^{a}	1.05
Oleic acid (18:1)	26.1^{b}	23.9^{b}	22.8^{ab}	17.4^{a}	1.85
Linoleic acid (18:2 (n-6))	17.5^{a}	30.6^{b}	35.4^{ab}	25.4^{a}	3.83
Arachidic acid (20:0)	0.7^{a}	2.2^{b}	2.4^{ab}	1.3^{a}	0.40
Eicosenoic acid (20:1)	0.3^{a}	1.5^{b}	0.75^{a}	n/d	0.33
Eicosatrienoic acid (20:3)	n/d	n/d	n/d	0.42	0.11
Arachidonic acid (20:4 (n-6))	5.0^{b}	2.3^{a}	4.2^{a}	13.1^{b}	0.36
Ecosapentanoic acid (20:5 (n-3))	n/d	1.6^{b}	0.74^{a}	n/d	0.38
Behenic acid (22:0)	n/d	n/d	0.4^{a}	0.64^{a}	0.16
Docosapentanoic acid (22:5 (n-3))	0.6^{a}	1.3^{b}	1.1^{a}	1.6^{a}	0.21
Docosahexanoic acid (22:6 (n-3))	2.0^{a}	6.4^{c}	6.1	5.8^{a}	1.02
Lignoceric acid (24:0)	n/d	n/d	n/d	0.52	0.13
Nervonic acid (24:1)	n/d	n/d	n/d	0.55	0.14
Total saturated fatty acids	47.8^{b}	29.4^{b}	27.3^{a}	34.1^{a}	4.61
Total unsaturated fatty acids	52.2^{ab}	70.6^{b}	72.7^{ab}	65.9^{a}	4.97
Total monounsaturated fatty acids	27.1^{ab}	28.3^{b}	25.1^{ab}	19.6^{a}	1.92
Total PUFA n-3	2.6^{a}	9.3^{c}	7.7^{b}	7.4^{a}	0.44
Total PUFA n-6	22.5^{ab}	33.0^{b}	39.6^{ab}	38.4^{a}	3.98
n-6 :n-3 ratio	8.6^{b}	3.5^{a}	5.12^{a}	5.19^{a}	0.76
U:S ratio	1.1^{a}	2.4^{c}	2.7^{c}	1.9^{b}	0.32
P:S ratio	0.5^{a}	1.4^{b}	1.73^{ac}	1.3^{b}	0.23

^{a,b,c}Values with different superscripts within rows are significantly different (P<0.05), n/d: not detected, S: Saturated, U: Unsaturated, P: Polyunsaturated. SEM: Standard error of means. BL: Baseline, CTRL: Control group, HFAR: High n-6: n-3 fatty acid ratio group, MFAR: Medium n-6: n-3 fatty acid ratio group, LFAR: Low n-6: n-3 fatty acid ratio group.

Table 6: Plasma total cholesterol (TC), triacylglycerol (TAG), LDL cholesterol (LDL-C) and HDL cholesterol (HDL-C) of rats on various diets

	Experimental diets			
	HFAR	LFAR	MFAR	CTRL
TC (mmol/L)	1.79±0.14^{b}	1.21±0.12^{a}	1.4±0.11^{a}	1.47±0.12^{ab}
TAG (mmol/L)	0.72±0.05^{b}	0.58±0.07^{a}	0.52±0.03^{a}	0.57±0.04^{a}
LDL-C (mmol/L)	0.41±0.03^{b}	0.3±0.05^{a}	0.33±0.03^{ab}	0.33±0.03^{ab}
HDL-C (mmol/L)	0.34±0.07^{b}	0.15±0.05^{a}	0.19±0.05^{a}	0.3±0.13^{ab}

Mean±SE. ^{a,b}Values with different superscripts within each row are significantly different (P<0.05). BL: Baseline, CTRL: Control group, HFAR: High n-6: n-3 fatty acid ratio group, MFAR: Medium n-6: n-3 fatty acid ratio group, LFAR: Low n-6: n-3 fatty acid ratio group

and HFAR rats accounting for 32%. Furthermore, LFAR still dominates the composition of n-3 PUFA with the amount of 10.5% when compared with other treatment groups. LFAR and MFAR group manifested considerably higher (P<0.05) concentration of Eicosapentanoic Acid (EPA), Docosapentanoic Acid (DPA) and Docosahexanoic Acid (DHA).

Muscle fatty acid profile: In muscle tissue, a significantly high (P<0.05) amount of total saturated fatty acid was detected in HFAR group with 47.8% compared to 29.4% in LFAR, 27.3% MFAR and 34.1% in CTRL. As appeared in the treatment diet, long chain n-3 fatty acid such as EPA, DPA and DHA existed in twofold concentration in LFAR in contrast to MFAR group. Although both DPA and DHA were detected in CTRL and HFAR groups, they were significantly (P<0.05) low for both treatment groups. The LFAR and MFAR groups showed a considerably high amount of n-6 fatty acid in comparison to CTRL and HFAR rats. This is mainly attributed to the high content of linoleic acid.

Plasma lipid profile changes: Table 6 summarizes total plasma cholesterol (TC), HDL-C, LDL-C and TAG concentrations in rats fed with different dietary supplementation. TC was significantly (p<0.05) lower in LFAR (1.21±0.12 mmol/L) and MFAR (1.4±0.11 mmol/L) groups when compared to HFAR group (1.79±0.14 mmol/L). Similar trends were observed in plasma TAG levels. Butter supplemented rats showed a significantly

higher (P<0.05) TAG compared to the CTRL and PUFA supplemented counterparts. The concentration of LDL-C and HDL-C was found significantly lowest (P<0.05) in LFAR group.

DISCUSSION

The present study describes fatty acid concentrations in plasma, red blood cell and muscle of *Sprague-Dawley* rats fed high fat diets rich in low, medium and high ratio of dietary n-3: n-6 PUFA compared with a standard chow diet. The study was also set out to assess the effects of each manipulated diets on indexes of insulin sensitivity and lipid profile changes in rats. Intake of high fat diet supplemented with SFA in rats induced hyperinsulinemia and reduced insulin sensitivity with impaired glucose tolerance (Alsaif and Duwaihy, 2004; Buettner *et al*., 2004; Storlien *et al*., 2000). Similarly, it was found that high fat diet rich in SFA in HFAR group caused hyperinsulinemia, reduced insulin sensitivity but no evidence of glucose intolerance unlike the n-3 and n-6 PUFA supplemented rats in LFAR and MFAR groups. This shows the compensatory insulin release due to significant insensitivity to insulin in HFAR group that increase glucose uptake into insulin-target tissue cells. After 22 weeks of dietary intervention, LFAR and MFAR rats showed no significant difference in terms of insulin sensitivity compared to the baseline rats. Similarly, it has been shown that the detrimental effect of SFA is prevented by adding long chain n-3 PUFA to SFA-rich diets (Alsaif and Duwaihy, 2004). Replacement of the portion of saturated fatty acid by long chain n-3 fatty acid appeared to reverse the effect of high saturated fatty acid which induced insulin resistance in rats (Storlien *et al*., 2000).

In agreement with our observation, Lee *et al*. (2006) reported that rats fed high saturated fat diet derived from lard and coconut oil demonstrated hyperinsulinemia with absence of change in glucose AUC compared to chow fed matches. Similarly, Madsen *et al*. (2002) observed presence of hyperinsulinemia in high fat fed rats in the absence of difference in the plasma glucose level. Lack of change in glucose tolerance despite reduced insulin sensitivity might be explained by stage of insulin resistance, where the glucose uptake by muscle which is responsible for the majority of postprandial glucose uptake, might not be interfered as a whole.

Hyperinsulinemia and reduced insulin sensitivity had been observed in CTRL group after 20 weeks of dietary intervention when compared to baseline group. This might be explained by the effect of aging on insulin sensitivity which is associated with insulin resistance (Mathews *et al*., 2006). This effect can be overcome by the supplementation of n-3 and n-6 PUFA, where group LFAR and MFAR showed a relative good control in insulin regulation. This was supported by the result in Fig. 7, where the glucose-insulin index was significantly lower for LFAR and MFAR group and there was not a big variation from the baseline group.

The difference in insulin sensitivity among HFAR and n-3 and n-6 PUFA supplemented LFAR and MFAR rats could be explained by the differential influence of dietary fats on insulin action. Composition of fatty acids in plasma and body tissues showed that insulin resistance is related to a specific pattern of fatty acids with a high content of SFA (mainly palmitic acid) and a low concentration of PUFA (mainly n-3 and n-6 PUFA) (Blaak, 2007; Vessby *et al*., 2002). PUFA particularly the long chain n-3 fatty acids were postulated to affect insulin sensitivity by incorporating into cell membranes and increasing membrane fluidity (Storlien *et al*., 2000; Cazzola *et al*., 2004). This in turn resulted in increased insulin receptor activity and improved in insulin signaling cascade required for the proper functioning of insulin. Furthermore, PUFAs are also known to function as a metabolic fuel repetitions through suppression of genes involved in lipid synthesis and upregulation lipid oxidative genes (Clarke, 2001). Enhancing lipid oxidation by dietary PUFA avoids lipotoxicity mediated insulin resistance by avoiding net positive energy balance (Lewis *et al*., 2002). Similar to the observation noted by Vessby *et al*. (2002) and Blaak (2007), in the current study evidence of insulin resistance was observed in butter supplemented (HFAR) rats unlike CTRL, n-3 and n-6 PUFA supplemented rats.

Several studies have investigated the influence of fatty acid composition of the diet on the fatty acid profile of skeletal muscle and red blood cell (Martín de Santa Olalla *et al*., 2009; Baur *et al*., 1999, 1998, 2000; Murphy *et al*., 2007). Skeletal muscle plays an important role in the energy metabolism of body including insulin-stimulated glucose uptake and fatty acid oxidation.

In the present study, fatty acid profile of plasma, red blood cell membrane and skeletal muscle in HFAR rats revealed the presence of high amount of saturated fatty acid mainly attributed to elevated concentrations of palmitic and stearic acid due to butter intake (Table 3-5). Likewise, fatty acid composition of skeletal muscle lipids reflects the fatty acid composition of the diet in healthy men and women (Andersson *et al*., 2002) which has been related to peripheral insulin sensitivity (Clore *et al*., 1998) and obesity (Kriketos *et al*., 1996) in numerous human populations.

The significantly high amount of long chain n-3 fatty acid concentration was detected from samples of rats fed with PUFA-supplemented diet as a whole and LFAR treated rats in particular compared to HFAR and CTRL groups.

The result of plasma lipid analysis revealed the distinct effect of dietary fatty acids on plasma TC, LDL-C, HDL-C and TAG. It was observed that LFAR and MFAR diets had significantly lower TC, HDL-C and TAG when compared

to HFAR rats. The lipoprotein data obtained in our study, agrees with the published literatures which showed that n-3 and n-6 PUFA supplementation particularly long chain n-3 fatty acid decreased TC in rats (Gaiva *et al*., 2003; Hong *et al*., 2003; Mohamed *et al*., 2002; Morgado *et al*., 2005). Concurring to our findings, various authors had also reported lowered concentration of HDL-C in response to fish oil supplementation (Morgado *et al*., 2005; Gaiva *et al*., 2003 Hong *et al*., 2003). In our study we also observed the plasma TAG showed suppressive effects of n-3 fatty acid which has been consistently reported in both animal and human studies using fish oil (Hatahet *et al*., 2003; Montoya *et al*., 2002; Gaiva *et al*., 2003). Dietary intervention studies in both human and animals indicated that fat sources containing saturated fatty acids such as butter, lard or coconut oil are shown to elevate TC, TAG and LDL-C with minimal or absence of effect in HDL-C (Hayes, 2000; Nicolosi, 1997; Mohamed *et al*., 2002; Montoya *et al*., 2002). A similar situation was observed in our finding where unlike TC, TAG and LDL-C concentration, the difference of HDL-C between control and butter supplemented rats was narrow.

Overall, in the present study 10% dietary fat supplementation from butter showed an evidence of insulin resistance along with alteration in plasma lipid profile in *Sprague-Dawley* rats. However, supplementation of high dietary fat with n-3 and n-6 PUFA in the current study showed a delay in the onset of insulin resistance and displayed favorable plasma lipid profile.

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